Molecular detection, monitoring and modulation of antigen specific immune responses

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy by

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Ce modeste pavé
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Abstract

Stem cell transplantation (SCT) represents the only curative treatment option for leukaemia. The bone marrow or peripheral blood stem cells transferred in the transplant procedure restore immune functions, allowing the targeting of infected cells or cells expressing tumour antigens. Cytotoxic T lymphocytes (CTL), key mediators of antigen specific killing, were investigated in the context of cytomegalovirus (CMV) infection or chronic myeloid leukaemia (CML).

CMV infection after SCT in the absence of effective immunological control or antiviral therapy is a significant cause of morbidity and mortality. HLA-A*0201 tetramer reagents were prepared with three candidate peptides and used to test healthy donor and SCT patient samples. Only T cells binding to the tetramer made with the predominant pp65 epitope (AE42: 495-503) were found for HLA-A*0201 healthy individuals and patients after SCT and correlated with the estimation of the responding T cell population to this peptide as measured by interferon-γproduction. Parallel tetramer and viral load monitoring of patients at risk of CMV infection after SCT highlighted an inverse correlation between the state of replication of the virus and the number of CMV specific CTL.

Presentation of a CML tumour specific peptide epitope in the context of HLA-A*0301 was demonstrated both at the surface of tumour positive cells lines and patient cells. HLA/peptide tetramer reagents were prepared with this peptide and used to screen CML patient samples. Low frequencies of peptide specific CTL were detected in some patients and could be stimulated *in vitro*.

While donor lymphocytes infusions would improve CTL responses after SCT, they also carry the risk of graft versus host disease and may not comprise an effective CTL population targeted to the antigen of interest. A better outcome may be obtained by infusion of enriched and or expanded specific T cell populations. The enrichment, stimulation and expansion of CTL with HLA/peptide tetramer or modified tetramer complexes was examined, using the HLA-A*0201/CMV CTL response as a model.

The use of HLA/peptide tetramers to monitor recipients of SCT has implications for the improvement of the treatment of CMV after SCT and the definition of targets and criteria for effective adoptive transfer. Furthermore, the use of HLA/peptide tetramers could be investigated in the assessment of anti-tumour CTL responses, in

enhancing existing responses and possibly in inducing primary immune responses to viral pathogens and to tumours.

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Abbreviations

The listed abbreviations have been used throughout this thesis and each citation was spelled in full on first appearance.

ANRI	Anthony Nolan Research Institute
ANT	Anthony Nolan Trust
APC	allophycocyanine
APS	ammonium persulphate
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
BD	Becton Dickinson
BSA	bovine serum albumin
bsp	biotinylation substrate peptide
CD	cluster of differentiation
CDR	complementary determining regions
cDNA	complementary deoxyribonucleic acid
CFSE	carboxy-fluorescein diacetate succinimidyl ester
CI	confidence interval
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CTL	cytotoxic T cell
Cy 5.5	cyanine 5.5
DC	dendritic cell
DLI	donor leucocyte/lymphocyte infusion
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulphoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DTT	DL dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay

ELIspot	enzyme linked immuno-spot
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
F-moc	fluoremethoxycarbonyl chemistry
Fos	Foscarnet
FPLC	fast protein liquid chromatography
Gan	Ganciclovir
GrB	granzyme B
GvHD	graft versus host disease
GvL	graft versus leukaemia
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
ICAM	intercellular adhesion molecule
ICOS	inducible co-stimulator
IE	immediate early
IFN	interferon
IL	interleukin
IPTG	isopropylthio-β-D-galactoside
IS	immunological synapse
ITAMs	immunoreceptor tyrosine based activation motifs
LFA	lymphocyte function-associated antigen
M	molar or moles per litre
MHC	major histocompatibility complex
MPa	mega Pascal (pressure units)
MWCO	molecular weight cut off
NBT	nitroblue tetrazolium chloride
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridiidin chlorophyll protein
pH	concentration of hydrogen ions in solution
PHA	phytohemagglutinin
PMSF	phenylmethylsulphoxide
pNPP	p-nitrophenyl phosphate

pp	phosphoprotein
RFH	Royal Free Hospital
RNA	ribonucleic acid
RPMI 1640	medium for lymphoid cell culture developed at the Roswell Park Memorial Institute
RT	room temperature
SCT	stem cell transplantation
SDS-PAGE	sodium dodecyl sulphate poly-acrylamide gel electrophoresis
sfu	spot forming unit
SP	single positive
TAP	transporter associated with antigen processing
TCD	T cell depletion
TEMED	N-N-N'-N' tetramethylethylene diamine
TNF	tumour necrosis factor
U	unit
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo 4-chloro 3-indolyl-β-D-galactosidase

Chapter I
Introduction

The immune system

The physical barriers and the cellular and molecular components that collectively defend an organism or host against disease constitute the immune system. The different components of the immune system have evolved and adapted to face a pathogen rich environment.

The immune system can be seen as a layered defence system, with first of all the presence of physical barriers such as the skin, mucosa and their protective linings as well as the digestive fluids represent the first obstacle to the entry of a pathogen. When this first barrier has been overcome, pathogens then face a series of cellular and soluble components, which have evolved to recognise characteristics that are common amongst pathogens and constitute the inherited arm of the immune response that can function without prior exposure of a host to the pathogen. This arm of the immune response is also named innate, and constitutes the first active line of defence that a pathogen encounters upon entry into the host tissues. The second active layer of defence consists of the specific targeting of the pathogen for destruction, as well as the destruction of cells that have been infected and harbour this pathogen, in order to avoid both infection and spreading. This requires a previous exposure to the pathogen that can thereafter be identified and will induce a swifter and stronger response to a repeat infection with the same pathogen. This type of response is named adaptive as it has the ability to conserve a memory of previous infections and can generate an improved response with time.

The major active components of the adaptive arm of the immune response originate from haematopoietic lymphoid progenitor cells in the bone marrow. Lymphoid precursors give rise to two progenies that are distinguished firstly by their location of maturation and by their specific receptor present at the cell surface. The differentiated cells or lymphocytes can be detected in peripheral blood or tissues after they have matured in the bone marrow (B lymphocytes) or in the thymus (T lymphocytes). B and T lymphocyte cell surface receptors enable the specific recognition

of a portion of the pathogen (or antigen) and are named respectively B cell receptor and T cell receptor (TCR). These receptors differ in structure and in expression as the B cell receptor is produced both as a transmembrane glycoprotein and as a soluble molecule (also named immunoglobulin or antibody) that binds antigen and elicits humoral effector functions, while the TCR only exists as a membrane bound molecule that elicits cellular effector functions. The functions of B lymphocytes upon pathogen recognition are to proliferate and to produce antibody molecules that can bind extracellular pathogens and mediate their elimination by phagocytosis or complement mediated lysis. By comparison, T lymphocytes recognise pathogen infected cells and will proliferate, secrete soluble molecules (or cytokines) that will provide "help" for the activation of B cells or can directly mediate killing (cytotoxicity) of the infected cell.

The recognition of pathogens by B or T cells occurs through specific binding of the B cell receptor or the TCR respectively. Immunoglobulins and TCRs are highly variable molecules, with the specificity concentrated in the region that binds to antigen. Both these receptor types are generated by random rearrangement of V, D and J gene segments and result in the production of an immunoglobulin molecule for B cells or of an $\alpha\beta$ or $\gamma\delta$ heterodimer for T cells. The majority of T cells present in peripheral blood express the $\alpha\beta$ heterodimer. These molecules were shown to bind foreign antigens when the latter were presented in peptide form refolded with major histocompatibility complex (MHC) proteins present at the cell surface (Zinkernagel and Doherty, 1974). There are two major distinct classes of MHC molecules: MHC class I molecules (or Human Leucocyte antigen, HLA-A, B and C) that are expressed on almost all nucleated cells and MHC class II molecules (or Human Leucocyte antigen, HLA-DR, DP and DQ) that are expressed mainly on cells with immunological functions such as dendritic cells (DC), B cells and macrophages. However, the expression level of MHC class I and MHC class II at the cell surface can vary. Antigens presented in the context of MHC class I or MHC class II molecules can be recognised by CD8 or CD4 T cells with cytotoxic or helper functions respectively.

To appreciate fully the physiological role of these cells, it is possible to analyse what happens when they are absent. Such deficiencies may be genetic, as is the case of the bare lymphocyte syndrome when cells lack expression of MHC class I and class II and in Omenn syndrome, when a defect in the recombinase genes prevents the development of B and T cells. Deficiencies can be induced, as in acquired immuno-deficiency syndrome (AIDS) and in patients whose immune systems are ablated during conditioning for allogeneic stem cell transplants. In this latter case, the recovery of the

immune system occurs after the transfer of donor haematopoietic stem cells and any residual mature T cells remaining in the stem cell graft. Reconstitution of the immune system is relatively slow in transplanted patients and varies depending on their age and the extent of T cell depletion. As a consequence, they may suffer a prolonged period of immunodeficiency when they are susceptible to infections and to relapse of the underlying malignancy if it has not been entirely ablated by treatment. The aim of this thesis is to follow specific immune responses in these patients with the overall goal of using specific T cells in adoptive immunotherapy during such prolonged periods of immunodeficiency. These studies have been first developed and have been practical in the context of MHC class I molecules and are therefore restricted to cytotoxic T cell responses. In many cases, these are appropriate cells to be given for adoptive immunotherapy since their function is to eliminate virus infected cells or residual tumour cells.

Antigen processing and presentation

In order to be recognised by T cells as self or foreign, proteins need to be processed in a manageable form at the cellular level so that only a portion of the antigen in the form of a peptide can be linked to MHC molecules and bind the TCR. All peptides binding to MHC molecules were shown to originate from the cytosolic cell compartment (Townsend and Bodmer, 1989). While MHC class II molecules bind peptides originating from extracellular pathogens that enter the cell within endosomal or lysosomal vesicles (Brodsky and Guagliardi, 1991). The protein fragments binding to MHC class I molecules originate in the majority of cases from viruses or intracellular bacteria, although peptides may also originate from other sources such as aggregated intracellular proteins. In this chapter, emphasis will be put more specifically on MHC class I processing and presentation pathway, as it is of relevance to the focus of this PhD thesis.

MHC class I structure

MHC class I molecules are heterodimeric molecules composed of a membrane spanning α chain glycoprotein or heavy chain bound non covalently to β2-microglobulin, which does not have any transmembrane or intracellular moieties. The α chain folds into three distinct domains: α1, α2 and α3 (see schematic representation Figure I.2). The α3 domain and β2-microglobulin show similarities in amino acid sequence with immunoglobulin constant domains and have similar folded structures. The α1 and α2

domains fold together into a single structure consisting of two segmented α helices lying over a sheet of eight antiparallel β strands to create a groove. Inside the groove formed by the refolded MHC class I molecule, a cluster of tyrosine residues common to all MHC class I molecules can form hydrogen bonds to the amino terminus of an 8 to 10 amino acid long peptide. Typically each MHC class I molecule accommodates a 9 amino acid long peptide that fits within the binding groove, which forms specific spaces or pockets available for the binding of each amino acid, numbered from 1 at the amino terminus to 9 at the carboxy terminus. A second cluster of tyrosine residues forms hydrogen bonds and ionic interactions with the peptide backbone at the carboxy terminus and with the carboxy terminus itself. Therefore the peptide is bound in an elongated conformation across the groove with tight binding at either end (or peptide anchor sites); with binding of the inner part of the peptide being more lose or flexible to accommodate the different peptide lengths (Stern and Wiley, 1994; Bouvier and Wiley, 1994; Rammensee, 1995). Both binding clusters are invariant between MHC class I molecules and are thought to be the main stabilising contacts for MHC/peptide interactions. However MHC molecules are highly polymorphic and the main differences between MHC variants are found at certain sites within the peptide binding groove and result in different amino acid involvement at key peptide interaction sites (such as anchor sites) in the different MHC variants (Albert *et al*, 2002). The anchor positions may differ between different MHC molecules, however they are similar for all peptides binding to the same MHC molecule. Consequently, different MHC variants will bind different peptides. Additionally, the peptide anchor residues that bind the same MHC molecule will not necessarily be identical, although they will have similar properties (for example, they will all be large hydrophobic residues such as Valine, Leucine or Isoleucine).

MHC class I processing and presentation

In the last decade, the detailed mechanisms that lead from pathogen entry into the cytosol of the infected cell to the MHC class I presentation of a peptide from that same pathogen have been elucidated (Germain, 1994; Lechner and Cresswell, 1996; Pamer and Cresswell, 1998). This process is summarised in Figure I.1. Proteins of pathogen origin are marked by ubiquitin and targeted to a cytosolic proteolytic unit named the proteasome (Niedermann *et al*, 1999). The cleaved peptides resulting from proteasome processing are then transported from the cytosol to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing complex or TAP.

Partially folded MHC class I molecules linked to calnexin are present in the ER and are chaperoned towards the TAP complex by calreticulin and Erp57 where they bind to tapasin (Sadasivan *et al*, 1996, Li *et al*, 2000). There, peptides of 8 to 10 amino acids in length can bind to MHC class I molecules and form a stable MHC/peptide complex providing the affinity of the peptide for the MHC molecule is sufficient. These refolded MHC class I/peptide complexes are then transported through the Golgi apparatus and onto the cell surface where they are presented and constitute specific ligands for antigen restricted T cells.

In order to target pathogens and pathogen infected cells, T cells must differentiate between self proteins refolded with an antigenic peptide or refolded with a self peptide by screening the surface of cells in the body. The mechanisms that account for T cell receptor diversity and that allows T cells to differentiate between self and non-self (i.e. pathogen derived) peptides occur in the thymus. These processes are known to be composed of three major stages: T cell receptor rearrangement followed by positive and negative selection (reviewed in Starr *et al*, 2003).

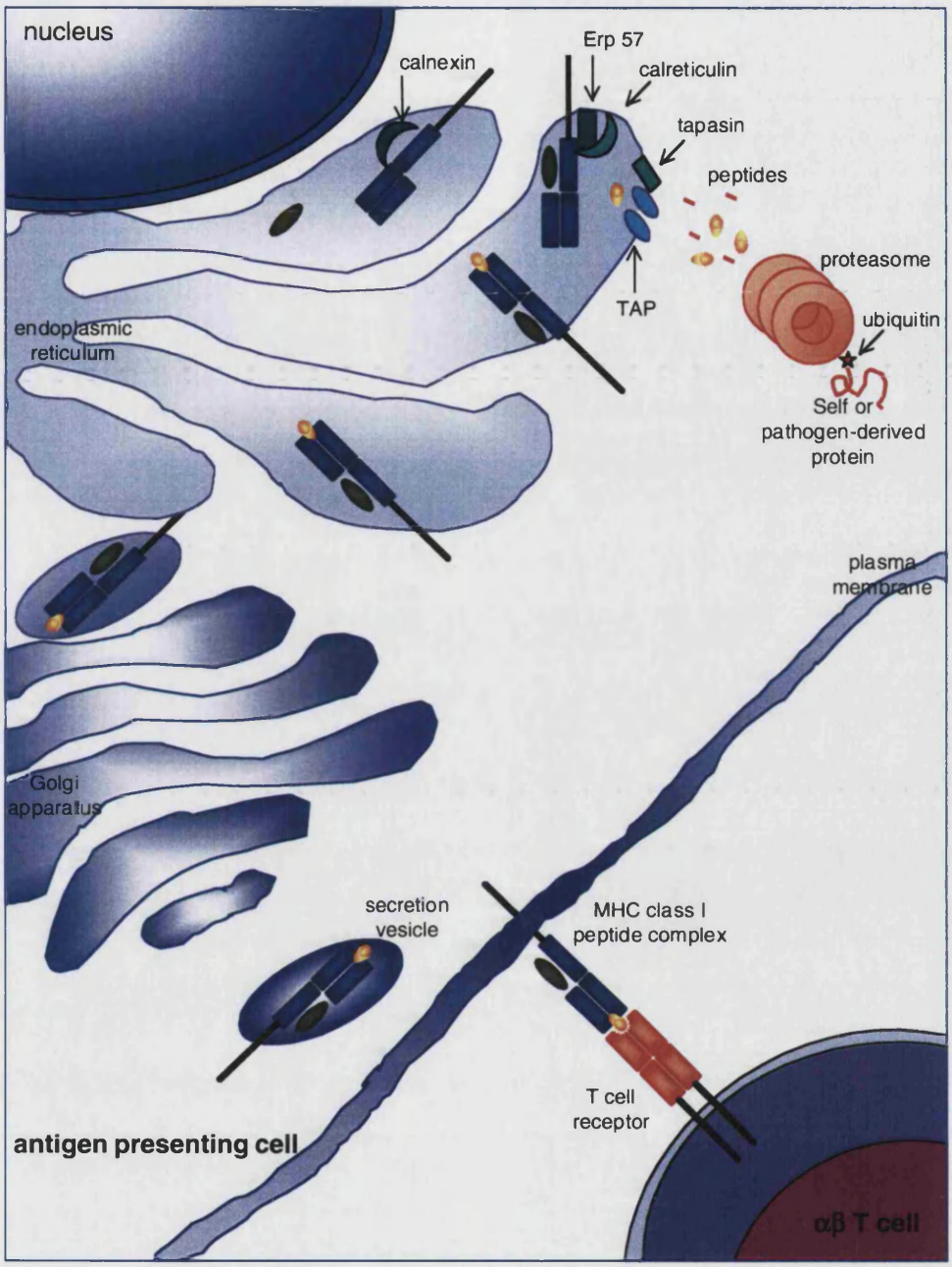

Figure I.1 MHC class I processing and presentation

The MHC class I processing and presentation pathway is restricted to cytosolic proteins that may originate from self or intracellular pathogens. Cytosolic proteins bound to ubiquitin are targeted to the proteasome where they are cleaved into peptide fragments. These fragments are then chaperoned to the transport associated protein (TAP) system composed of TAP1 and TAP2 protein sub-units, which mediates their transfer to the endoplasmic reticulum (ER). Unfolded MHC class I heavy chain proteins are released in the ER after synthesis and are chaperoned by calnexin until they refold with β-2 microglobulin. Once the MHC class I-β-2 microglobulin complex is formed, it is released by calnexin and chaperoned towards the TAP complex by the calreticulin and erp57 proteins and "docks" near the TAP complex via binding to tapasin. There, proteins transported in to the ER from the cytosolic compartment can bind to the pre-formed MHC-β-2 microglobulin complex if the peptide is from 8 to 10 amino-acids long and bind the complex with enough affinity to form a stable molecule. The refolded MHC class I-β-2 microglobulin/ peptide complex is then exported through the Golgi apparatus to be secreted and expressed at the cell surface where it can then be presented to T lymphocytes.

Professional antigen presentation: dendritic cells

Dendritic cells (DCs) share with their close relatives the macrophages the ability to specifically recognise and ingest pathogens. Other extracellular antigens can also be taken up non-specifically by macropinocytosis, a process in which large volumes of fluid surrounding the cells are engulfed. Unlike macrophages, the primary function of DCs is not to destroy, but to process and carry pathogen antigens from the site of infection to peripheral lymphoid organs where they can be presented to T lymphocytes (for reviews on dendritic cells, see Banchereau *et al*, 2000; Guermonprez *et al*, 2002).

DCs are leucocytes that originate from $CD34^+$ haematopoietic progenitor cells in the bone marrow. $CD34^+$ cells constitute the precursors of common myeloid and lymphoid progenitors, which have been shown to differentiate into DC precursors when they reach the peripheral blood circulation. DC precursors further differentiate in several types of immature DCs (which have not yet encountered antigen) characterized by their morphology, phenotype and location. They constitute the Langerhans, interstitial, monocyte-derived or plasmacytoid type of DCs after exposure to GM-CSF and FLT-3 *in vivo* and locate to peripheral tissues. (Sallusto and Lanzavecchia, 1994; Strunk *et al*, 1997; Bjorck, 2001). Immature DCs are characterized by the synthesis of large amounts of MHC class II molecules that remain sequestered in late endosomes and lysosomes with internalized antigens, therefore these do not present antigens efficiently to T lymphocytes. For efficient antigen presentation to occur, a process of maturation is necessary. *In vivo*, DC maturation is triggered by the cells sensing the presence of infectious agents such as microbial products or viral pathogens through specific cell surface receptors and by the triggering of tumour necrosis factor (TNF) receptors sensing local inflammation. The receptors binding to infectious agents specifically recognize components such as for example mannose, liposaccharide (LPS), which are both components of the bacterial membrane, and CpG DNA (specific deoxynucleotide motif characteristic of bacterial DNA) respectively recognized by the lectin-mannose receptor, the Toll-like receptor 4 (TLR4) and TLR9. (Poltorak *et al*, 1998; Qureshi *et al*, 1999; Hemmi *et al*, 2000; Geijtenbeek *et al*, 2000). Furthermore, the presence of double stranded RNA characteristic of some viral infections triggers the production of TNF-α, an inflammatory cytokine, which also constitutes a signal for DC maturation. Likewise *in vitro*, DCs can be matured by exposure to LPS, inflammatory cytokines including TNF-α and IL 4, as well as by CD40 ligation. (Sallusto and Lanzavecchia, 1994; Romani *et al*, 1996; Caux *et al*, 1994).

While MHC class II molecules accumulate in large numbers in endosomes and lysosomes, this is not the case for MHC Class I molecules, which bind cellular peptides in the ER as part of the

endogenous pathway of antigen presentation. The expression of MHC class I molecules is upregulated upon DC maturation, and there is evidence that this may drive the formation of the immunoproteasome. DCs are able to present peptide antigen in the context of MHC class II or MHC class I for presentation to $CD4^+$ and $CD8^+$ T lymphocytes respectively. To present foreign antigens in the context of MHC class I, they specialize in forming MHC class I-peptide complexes by the "exogenous" or "cross-presenting" pathway (Albert *et al*, 1998). This property was first described by experiments examining the nature of MHC restriction during T lymphocyte priming. Minor histocompatibility antigens were capable of being transferred from the cells expressing these antigens to host antigen presenting cells (Bevan, 1976). This type of presentation was named "cross-priming" to differentiate it from direct T cell activation by the actual cells expressing the minor antigens. Two different routes of cross-presentation have been described: the first is termed cytosolic as antigens are thought to be exported from the endosomal compartment into the cytosol, from where they follow the endogenous antigen processing pathway (TAP dependent, as described previously); the second route involves loading of peptide antigens onto MHC class I molecules in the endosomal pathway or directly on the cell surface (TAP independent). Cross-priming by DCs is involved in many types of immune responses, including these to tumours, viruses, grafted tissues and in some cases against self (Albert *et al*, 1998; den Haan *et al*, 2000). The cross-presentation of exogenous antigens is an important mechanism for $CD8^+$ T lymphocyte priming in infectious diseases as this allows the induction of responses to pathogens that do not directly infect antigen presenting cells and/or to pathogens that suppress the functions of antigen presenting cells.

While maturing, DCs simultaneously migrate from the peripheral tissue where the antigen was encountered into the paracortical region of the nearest draining lymph node where their long "dendritic" processes maximize the opportunities for T lymphocyte interaction. Mature DCs are then characterized by the redistribution of MHC class II molecules coupled to antigenic peptides from the intracellular compartments to the plasma membrane where they accumulate. The expression of MHC class I, co-stimulatory CD80 and CD86, as well as adhesion CD48 and CD58 molecules is also upregulated, which collectively with MHC class I and Class II expression confer exceptional capacity for T lymphocyte stimulation. (Inaba *et al*, 1998; Turley *et al*, 2000; Inaba *et al*, 2000; Bousso and Robey, 2003). DCs specialize in the stimulation of naïve T lymphocytes and the development of the adaptive immune response (further described in the following paragraphs). However, the outcome of dendritic cell stimulation can vary dramatically in its effect depending on the type as well as the quantity and quality of the signal the DC receives. Hence mature but quiescent DCs, or resting DCs targeted

with low doses of soluble antigen were shown to induce peripheral tolerance, in part through the induction of peptide-specific regulatory T cells (Dhodaphar et al, 2002; Steinman et al, 2003). Therefore DCs, while being the trigger of antigen specific T lymphocyte immunity, are also key to the maintenance of peripheral tolerance to self. This is particularly important due to the fact that some self protein for which tolerance is required may not be included in the process of thymic selection (if they are absent from the thymic environment) to remove self-reactivity, and that chronic reactivity to most harmless environment proteins must not develop. The activation of mature dendritic cells is critical to the induction of primary T lymphocyte responses, but cal also trigger memory type responses along with other non-specialised antigen presenting cells. The focus of this thesis will be centered more specifically on the generation of $CD8^+$ peptide-specific cytotoxic T cell responses. The requirements for T lymphocyte recognition and stimulation will be described in further detail in the following paragraphs.

T cell development

Common lymphoid progenitors expressing CD34 migrate from the bone marrow to the thymus near the cortico-medulary junction (Berenson *et al*, 1988; Terstappen *et al*, 1991). These cells develop and differentiate in the thymic microenvironment through cytokine signal and interaction with stromal cells. The relative expression of CD4 and CD8 markers is used to refer to the different stages of thymocyte differentiation: the first stage produces double negative 1 (DN1) type cells that are also $CD44^+$ and $CD25^-$. As these cells migrate towards the subcapsular thymic epithelium, they up-regulate CD25 (DN2) then down-regulate CD44 (DN3). At this point, receptor gene rearrangements occur and direct thymocytes to the γδ or the αβ differentiation pathway (Mallick *et al*, 1993; von Boehmer and Fehling, 1997). When productive TCR β rearrangement and successful pairing with pre-Tα (composed of an invariant α chain) occur, CD25 is then downregulated (DN4), followed by an extensive proliferation stage and with the expression of both CD4 and CD8 (double positive or DP) (Fehling and von Boehmer, 1997). The rearrangement of TCR α follows and multiple successive rearrangements can occur until a productive one is obtained. Each developing T cell α chain is then tested together with the β chain for recognition of self-MHC: this constitutes a positive selection process that will determine their survival. The peptide ligands involved in positive selection must have low affinity for the TCR and therefore will not be stimulatory for mature T cells (i.e. be antagonist peptides, Hogquist *et al*, 1994). Successful positive selection will then determine the down-regulation of either CD4 or CD8 and produce thymocytes that will recognise peptides bound to MHC class II or MHC class I and go onto become cytokine secreting cells or cytotoxic cells respectively (Basson *et al*, 1998; Singer *et al*, 1999). The second selection process or negative selection involves the clonal deletion of a given T cell if stimulatory ligands (foreign, agonist or antigenic peptide) are present in the thymus during development. This eliminates self-reactive responses to high affinity ligands (Kishimoto and Sprent, 1997; Zal *et al*, 1994). Both processes co-jointly allow only cells with functional TCRs that will not be self-reactive in the periphery to pass these checkpoints. As a result, less that 5% of thymocytes will successfully pass these two selection processes and migrate through the medulla before leaving the thymus.

T cell receptor composition, specificity and signalling

This paragraph will be focused on the composition, specificity and signalling of αβ T cells as they are of main interest in this thesis. By contrast, γδ T cells appear to have different antigen recognition properties and functions that will not be detailed here as they are outside the scope of the studies described in this thesis.

TCR composition and specificity

The structure of a number of TCR and MHC molecules has been determined by X-ray crystallography studies.

The TCR is composed of a glycosylated polymorphic αβ heterodimer, which is associated with a non-polymorphic membrane bound complex collectively known as CD3. Both the α and β chains of the TCR have an amino terminal region that shows homology to an immunoglobulin variable domain and carboxy terminal region that shows homology to an immunoglobulin constant domain. Each chain contains a hinge region near the cytoplasmic membrane that form a disulphide bond, and ends in transmembrane and short cytoplasmic tail fragments (see schematic representation, Figure I.2). Both chains are composed of two domains that resemble the variable and constant domains of immunoglobulins and the antigen binding site of the TCR has been described to consist of regions analogous to the complementary determining regions (or CDR) of immunoglobulins. T cell recognition is focused on peptides bound to class I MHC molecules composed of a transmembrane α chain that folds into three domains: α1, α2 and α3 (or heavy chain) and is non-covalently linked to β2-microglobulin.

Three CDR regions of the TCR have been shown to contact MHC molecules: CDR1, CDR2 and CDR3 (for a review on TCR structure, see Bentley and Mariuzza, 1996). The MHC is highly polymorphic, the majority of the diversity is located within the peptide binding groove and will not directly affect recognition by the TCR, which binding to MHC is restricted by its CDR1 and CDR2 loops. Most of the contact between the TCR and MHC that confers the peptide specificity for both complexes resides in the CDR3 domain of the TCR and the peptide binding motifs of the α1 and α 2 domains of the MHC heavy chain. The molecular recognition between the TCR and MHC/peptide complexes has been further characterised with X-ray crystallography studies of bound complexes, which allowed the visualisation of their binding pattern (Garcia *et al*, 1996; Garboczi *et al*, 1996) as well as their diagonal docking topology (Teng *et al*, 1998; Ding *et al*, 1998). Furthermore the MHC groove was demonstrated to be able to facilitate a degree of peptide binding flexibility to the TCR with 2 amino-

acids of the typically 9 forming the peptide anchored within the MHC and the others exposed for binding the TCR thus enabling relative spatial flexibility (Garcia *et al*, 1998). Binding of the TCR to MHC/peptide complexes leads to oligomerisation of TCR molecules and triggers activation (Reich *et al*, 1997), which is characterised by a complex cascade of downstream intracellular signalling events.

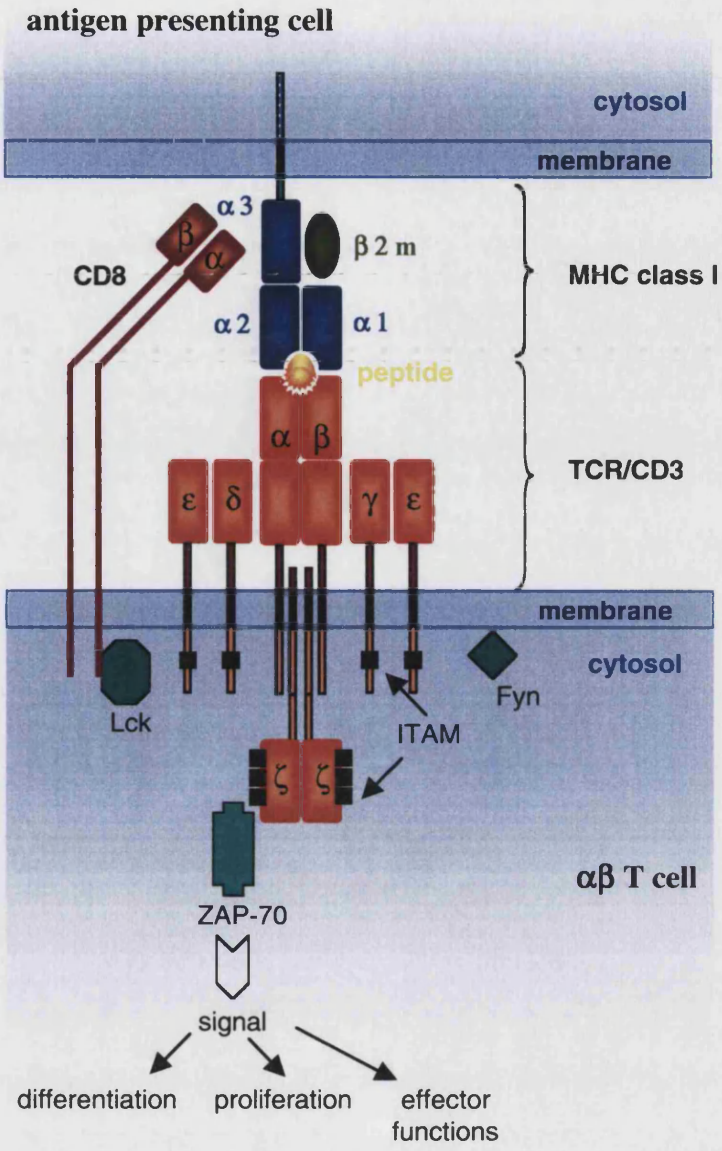

Figure I.2 αβ T cell recognition and signalling

The T cell receptor (TCR) is composed of the αβ heterodimer associated with four signalling chains: δ, γ and ε to form the TCR/CD3 complex. The TCR/CD3 complex is associated with a ζ chain homodimer, which possesses immunoreceptor tyrosine-based activation motifs or ITAMs. These are similar to the TCR/CD3 ITAMs. This complex binds specifically the major histocompatibility (MHC) class I-β-2 microglobulin/peptide complex in an antigen restricted manner. MHC binding induces the clustering of TCRs and the activation of receptor associated kinases such as Fyn or Lck. Binding of the α CD8 co-receptor sub-unit to the α 3 domain of MHC class I molecules brings Lck in close proximity to the TCR/CD3 complex and phosphorylates ITAMs, which in turn induce ZAP-70 phosphorylation and activation. This constitutes the first events leading to downstream signalling.

T cell signalling

The initial stages of T cell activation are summarised in Figure I.2. T cell activation was shown to be dependent on the binding affinity of TCR to the MHC peptide complex and on the kinetics of the interaction. This highlighted the facts that low affinity and high dissociation rates would not lead to downstream signalling and T cell activation although binding could occur (Matsui *et al*, 1994; Alam *et al*, 1996; Lyons *et al*, 1996; for a review, see Germain and Stefanova, 1999). Furthermore, interaction of the CD8 co-receptor with the α3 domain of the MHC class I heavy chains is important to triggering T cell activation as it will lead to the co-localisation and phosphorylation of Lck followed by that of ZAP-70 and marks early T cell activation events (Figure I.2). These last points serve to demonstrate the facts that TCR-MHC/peptide interaction alone may not be sufficient for efficient T cell activation. In fact full T cell activation requires a complex system involving multiple molecular recognition complexes and spatial segregation at the cell surface is now known to be involved. This was initially described and visualised at the cell surface by microscopic studies of what has since been named the immunological synapse or IS (Monks *et al*, 1998; Grakoui *et al*, 1999; Davis *et al*, 1999; for reviews see Krummel and Davis, 2002; Davis, 2002). The IS forms on contact of a T cell with its antigen presenting cell, initially by interactions between the lymphocyte function-associated antigen-1 (LFA-1) and the intercellular adhesion molecule-1 (ICAM-1) and between CD2 and CD58 respectively. Interestingly, these interactions effectively mediate initial binding of the T cell to an antigen presenting cell rather than TCR interaction. Once the initial contact has been established, TCR-MHC/peptide binding occurs and the CD2/CD58 complex segregate together with TCR/MHC and CD8 at the centre of the IS (Gao and Jakobsen, 2000). This effectively pushes LFA-1/ICAM-1 outwards as well as other much larger molecules such as CD43 and CD45 that can interact and influence T cell recognition without disturbing the binding of smaller molecules localised at the centre of the IS (Delon *et al*, 2001). These rearrangements can be visualised at areas of cell contact and form what appears like annular molecular clusters driven by changes in the T cell cytoskeleton structure (Valitutti *et al*, 1995; Dustin and Cooper, 2000). Therefore it is currently thought that these interactions collectively contribute to T cell recognition and signalling.

Additionally, there is recent evidence that co-stimulation can contribute to T cell recognition and can influence as well as regulate signalling to enhance or prevent T cell activation and proliferation (Wülfing *et al*, 2002; Egen *et al*, 2002, Holdorf *et al*, 2002).

Although prior evidence of the influence of a number of co-stimulatory molecules on T cell activation had been established, there is increasing data demonstrating that their contribution to signalling occurs through the IS.

T cell co-stimulation

T cell activation therefore requires the specific recognition of a MHC/peptide complex as described above. The co-stimulation of T cell responses involves the simultaneous interaction of molecules that are involved in modulating the subsequent response.

As previously mentioned, the direct involvement of CD4 or CD8 in MHC recognition and signalling point to them as being the major co-stimulatory molecules. However, a number of other molecules that can also influence T cell activation have been described more recently and these belong to the same family of molecules, act in a similar fashion and may even compete for the same receptor ligands. These molecules can be separated into two groups, depending on whether they enhance or suppress T cell activation therefore leading to anergy or death. Some of these co-stimulatory molecules and their ligands are represented on Figure I.3.

The main co-stimulatory molecules currently thought to promote T cell activation and clonal expansion (positive co-stimulation) include CD28, CD40L, 4-1BB and the recently described ICOS (inducible co-stimulator) (Andreasen *et al*, 2000; Liang and Sha, 2002). The opposite effect can also be seen by negative co-stimulation with a number of molecules and ligands, which can prevent T cell activation (anergy) or can induce programmed cell death. These are thought to be part of the control of the immune response as well as a safeguard from excessive or unwanted responses. They include CTLA-4, a CD28 analogue that directly competes with the same ligands CD80/86 also named B7.1/B7.2, and PD-1 (Wells *et al*, 2001; Greenwald *et al*, 2002).

CD28 is a co-stimulatory molecule that is traditionally thought to be enhancing significantly T cell activation by lowering the TCR threshold needed to generate a response (Bromley *et al*, 2001). CD28 is expressed on most T lineage cells, plasma cells and binds to the structurally related B7.1 (CD80) and B7.2 (CD86) glycoproteins that are expressed on peripheral blood monocytes and DCs. CD86 is also expressed at low levels on resting B and T cells. Upon T cell activation, the expression of CD80 and CD86 are increased rapidly, with CD86 reaching higher levels than CD80 (Linsley and Ledbetter, 1993; Linsley *et al*, 1994; Lenschow *et al*, 1996). However T cell activation also leads to CD28 up-regulation, but ligation leads to transient down-regulation.

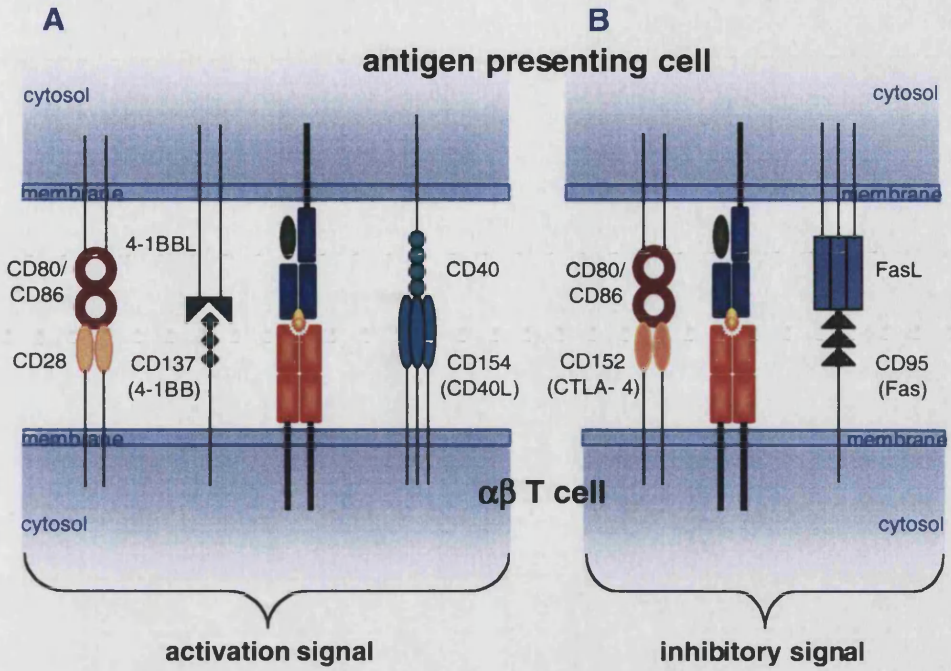

Figure I.3 Major co-stimulatory and inhibitory signals to αβ T lymphocytes

Representation of the molecular complexes currently thought to be the major co-stimulatory or inhibitory mediators of αβ T lymphocyte activation. Panel A summarises positive co-stimulatory signals mediated by binding to **CD28** (thought to constitute a major co-stimulatory signal necessary for naïve T cell priming), CD154 (also known as **CD40L**, an accessory co-stimulatory signal) and CD137 (also referred to as **4-1BB**, thought to be important for T cell survival and proliferation). Panel B summarises negative signals mediated by CD152 (also known as **CTLA-4**, in direct competition with CD28) and **CD95** (which induces the apoptotic pathway). Both these signals are involved in direct inhibition of the response and are part of the negative feedback mechanism resulting from T cell activation. The common names for these molecules are highlighted in bold and will be used in this thesis.

Binding of CD80 or CD86 to CD28 is considered to be the main co-stimulatory signal necessary to induce the activation of naïve T cells (or signal 2) co-jointly with MHC/peptide interaction (or signal 1). Once a naïve T cell becomes activated, it expresses a number of proteins that contribute to the co-stimulatory signal and help drive proliferation (clonal expansion) and differentiation.

The CTLA-4 (CD152) sequence closely resembles that of CD28, consequently, the CTLA-4 molecule directly competes for binding to the same ligands (CD80 and CD86) as CD28. It is expressed on activated, but not on resting T lymphocytes.

However, CTLA-4 binds these ligands 20 times more avidly and delivers a negative co-signal to T cells. This signal renders naïve T cells less sensitive to stimulation and limits the proliferation of activated T cells. This was demonstrated using a blocking CD152 antibody, which was able to induce an enhancement of T cell responses *in vitro* and *in vivo* (Kearney *et al*, 1995; Leach *et al*, 1996). Furthermore, CD152 deficient mice were shown to develop a fatal lymphoproliferative disorder, confirming the importance of the T cell inhibitory feedback by CTLA-4 (Tivol *et al*, 1995; Waterhouse *et al*, 1996).

CD40L (CD154) is also absent from the surface of resting lymphocytes, but is rapidly expressed upon activation, mostly on $CD4^+$ T cells but also on a small number of $CD8^+$ T cells and binds CD40. When expressed on $CD4^+$ T cells, it binds to CD40 on B cells and is required for secondary immune responses and germinal centre formation. When expressed on $CD8^+$ T cells, it induces antigen presenting cells to up-regulate CD80 and CD86, therefore stimulating further activation and/or proliferation (van Kooten and Banchereau, 1996; Gruss and Dower, 1995; Foy *et al*, 1996). Confirming the points described previously, the study of CD40L deficient mice revealed an increased susceptibility to parasite infections, therefore pointing to deficiencies in both cell mediated and humoral immunity (Noelle *et al*, 1996).

Similarly to CTLA-4 and CD40L, 4-1BB (CD137) is also absent from the surface of resting T cells, but is induced upon activation in T cells, B cells and monocytes (Schwarz *et al*, 1995; Pollok *et al*, 1994). Its ligand, 4-1BBL, is expressed on activated B and T lymphocytes and co-stimulates T cell growth; reciprocally, 4-1BB binding 4-1BBL co-stimulates B cells (Hurtado *et al*, 1995; DeBenedette *et al*, 1995).

Although the pathway that leads to programmed cell death by cross-linking CD95 (Fas) is not usually thought of as being part of a negative co-stimulatory system, it can be considered as such in this context, similarly to the range of molecules described previously, as it also plays a role in the feed-back mechanism induced after T cell activation. CD95 is expressed by activated lymphocytes, monocytes, neutrophils and fibroblasts, while its ligand (CD95L or FasL) is mainly restricted to T lymphocytes and is induced rapidly upon activation (Nagata and Golstein, 1995; Takahashi *et al*, 1994). Programmed cell death of activated lymphocytes occurs mainly through CD95 and is thought to be crucial to the maintenance of peripheral tolerance (Lynch *et al*, 1995; Takayama *et al*, 1995). Fas mediated killing also represents part of the T cell effector functions and will be described in further detail below.

Therefore it is becoming clear that signalling through the TCR may depend on the summation of all these potential co-stimulatory effects along with the affinity and

kinetics of the TCR-MHC/peptide interaction. These may collectively contribute to the rise, inhibition or elimination of T cell immune responses.

T cell activation

CD 69 is not expressed on resting peripheral blood lymphocytes but appears within 2 hr of TCR triggering, and therefore is one of the first molecules that becomes up-regulated at the cell surface. Consequently, detection of up-regulated CD69 expression constitutes an early marker of T cell activation (Hamann *et al*, 1993).

T cell effector function

Upon activation and differentiation into effector cells, the T cell subsets, $CD4^+$ or $CD8^+$, differ in their effector functions. The main aim of $CD8^+$ T cells is to induce cytotoxicity and eliminate pathogen infected cells. Processes involved in $CD8^+$ T cell effector function and cytotoxicity are detailed in the following paragraph.

Cytokine secretion

The activation of $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) induces the synthesis of soluble secretory molecules named cytokines. The two main effector cytokines that have roles in T cell clonal expansion and effector function are interleukin-2 (IL2) and interferon-γ (IFN-γ) respectively.

Upon T cell activation, IL2 synthesis is induced along with the α chain of the IL2 receptor (CD25). The IL2 receptor has different binding affinities to its ligand depending on the formation of a low affinity receptor composed only of the β and γ chains or of the high affinity receptor composed of α, β and γ chains. This system allows resting T cells to respond to high concentrations of IL2 whereas activated T cells can respond to low concentrations of IL2. IL2 binding triggers T cell progression through the cell cycle and can induce exponential divisions leading to clonal expansion and differentiation (Minami *et al*, 1993). As T cell activation induces both IL2 and IL2 receptor, it is therefore possible for a T cell to stimulate its own proliferation and differentiation as well as that of other activated T cells. Additionally, co-stimulation through CD28 is needed for the long-term induction of the IL2 receptor (Cerdan *et al*, 1995).

The release of IFN-γ by T cells upon T cell activation is one of the first effector functions that will have an effect on pathogen destruction. The main function of IFN-γ resides in macrophage and NK cell activation (Munoz-Fernandez *et al*, 1992; Stout and

Bottomly, 1989). It is also implicated in the induction of MHC class I and MHC class II molecules expression as well as the enhancement the antigen presenting functions mainly of professional antigen presenting cells such as DCs and macrophages as well as other somatic cells (Steimle *et al*, 1994). It achieves this by acting on proteasomes, which are IFN-γ inducible and the LMP components are altered to provide faster and more efficient processing of antigens. For both MHC class I and MHC class II the increased export to the cell surface means that more peptides will be presented. Both these effects will therefore contribute to and improve immune reactivity towards the pathogen infected cell.

Cell mediated cytotoxicity

Fas (CD95) mediated killing is the first mechanism of T cell mediated cytotoxicity and is also referred to as programmed cell death. The binding of tumour necrosis factor (TNF) family receptor CD95L to CD95 induces the trimerisation of the latter and regroups the cytoplasmic tails of CD95 that include motifs known as death domains (Nagata *et al*, 1996). These will bind to the death domain protein with the cystein protease caspase, which in turn activates a cascade of caspase enzymes and leads to the cleavage of the cytoplasmic inactive caspase-activable DNase (CAD). CAD then translocates to the nucleus where it cleaves DNA and leads to cell death (Henkart, 1994; Squier and Cohen, 1994; Medana *et al*, 2000).

The second and main mechanism by which cytotoxic T cells mediate killing is through the calcium dependent release of lytic granules upon recognition of an MHC/peptide antigenic target (for a review, see Trapani *et al*, 1999). These granules comprise two types of molecules with different effects, but that act synergistically to achieve effective cell killing. The first of these enzymes is a pore forming protein named perforin that inserts into the target cell where it breaks the integrity of the cytoplasmic membrane by forming pores (Griffiths and Argon, 1995). This allows for the cellular uptake of a second family of enzymes contained in the granules named granzymes. The principal component consists of the serine protease granzyme B (GrB), which acts by activating a caspase enzyme that leads to DNA degradation and apoptosis as described above.

Phagocytes then target cells undergoing apoptosis and ingest them, therefore eliminating both pathogen and infected cell.

αβ T cell differentiation and cytokine requirements

As described earlier, naïve $CD8^+$ T lymphocytes leave the thymus for the peripheral blood circulation. Their differentiation pathway can be separated into different stages that are characterised by the expression of cell surface marker and include: 1) naïve, before the T cell has encountered its specific MHC/peptide ligand; 2) memory/effector, after encountering specific antigen and migrating into the lymph nodes therefore acquiring a more rapid response potential to secondary exposure, clonal proliferative potential as well as effector functions; 3) effector/memory when the cell acquires cytotoxic properties while retaining a proliferative potential; 4) effector when the cell is mainly destined for cytotoxic function and has limited or no replicative potential. Each of these differentiation stages, or compartments, are represented on Figure I.4, together with the cell surface molecule expression, or markers, and secretory molecules characteristic of each stage. These markers include the common leucocyte antigen CD45, the co-stimulatory molecules CD27 and CD28 as well as the lymph node homing factor CCR7. The relative levels of effector and cytotoxic molecules released by these cells are also specific to the T cell differentiation stage (for a review, see van Baarle *et al*, 2002).

Naïve or unprimed T cells express CD45RA, the co-stimulatory molecules CD28 and CD27, as well as CCR7, but have no effector function. Upon TCR activation, CD45RA expression is lost and CD45RO expression becomes up regulated. The $CD45RO^+$ $CD27^+$ $CD28^+$ memory T cells have very high proliferative potential, but only become cytotoxic after re-stimulation (Hamann *et al*, 1997). After successful homing to the lymph nodes, CCR7 become down-regulated and further stimulation will then induce CD28 down-regulation (Azuma *et al*, 1993). Additionally, after prolonged stimulation, CD27 co-stimulation will induce further T cell proliferation, induce cytotoxic functions, enhance cytokine production, which will result in the irreversible down regulation of the receptor from the cell surface (Gruss and Dower, 1995; Kobata *et al*, 1995). The effector and cytotoxic pathways are now fully up regulated and effector T cell will re-express CD45RA. Although these cells were thought to be terminal effectors that would be irreversibly committed to this compartment and die after encountering antigen, there has been *in vitro* evidence that they may revert to less differentiated $CD45RO^+$ cells.

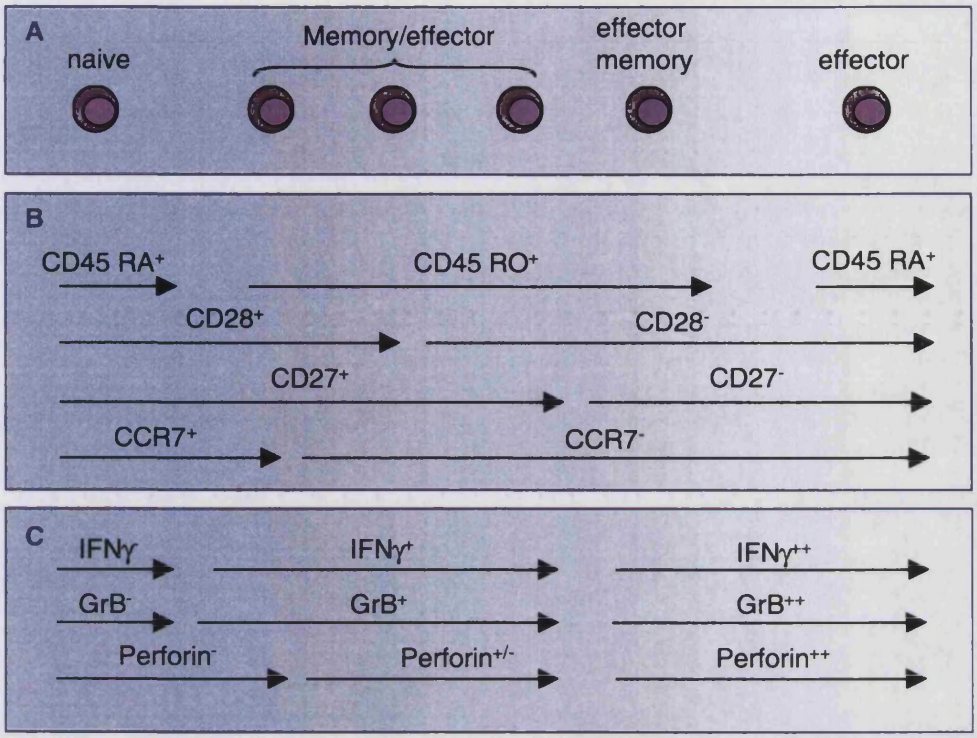

Figure I.4 αβ T lymphocyte differentiation: cell surface expression and effector function

Activation of $CD8^+$ αβ T lymphocytes results in differentiation of the cell characteristics and changes in the expression of cell surface molecules and the secretion of effector molecules. The general stages of $CD8^+$ T cell differentiation pathway is summarised in Panel A. Panel B summarises the expression pattern of markers of activation such as CD45, CD28 and CD27. CCR7 is a lymph node homing receptor that is expressed on cells that localise to lymph node (termed "central memory" in the case of memory cells). Panel C summarises the effector and cytotoxic properties of these cells. Collectively, the expression of these marker expression patterns and effector functions are characteristic of the level of differentiation of $CD8^+$ α β T lymphocytes and referred to as their phenotype.

The survival, proliferation and differentiation of CTLs are also dependent on the specific cytokine signals that these cells receive. As T cells can remain in peripheral blood for prolonged periods of time without specific antigen stimulation, the effect of cytokines explains their capacity to survive.

The four main cytokines involved in T cell survival and/or proliferation are IL7, IL12, IL2 and IL15 (reviewed in Marrack *et al*, 2000). IL7 is mainly implicated in T cell survival and in the cycling of resting T cells. Together with IL12, which is thought to provide a third activatory signal to naïve T cells, it contributes to the priming and

long-term maintenance of antigen specific T cells (Curtsinger *et al*, 1999; Schmidt and Mescher, 2002; Montagna *et al*, 2001). Although IL2, as described previously, induces very potent clonal expansion of activated T cells, it is also involved in the feedback mechanism controlling the immune response and is therefore capable of activating the programmed cell death pathway. Similarly, IL15, which can bind to the same β and γ receptor chains as IL2 but has its own specific IL15 receptor α chain, is also capable of inducing clonal expansion but cannot induce the programmed cell death pathway (Waldmann *et al*, 2001). There is also recent evidence that the presence of IL15 in the generation of primary and memory antigen specific T cells can increase the overall magnitude of the response (Schluns *et al*, 2002).

Haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (SCT) constitutes the standard and only curative treatment option for many haematological malignancies. These are clonal diseases thought to derive from a single cell in the marrow or peripheral lymphoid tissue that has undergone one or several genetic alterations that leads to malignant transformation.

The general principal of SCT consists of the destruction of cancer cells by a combination of radiation therapy and chemotherapy (or conditioning regimen), followed by bone marrow rescue and reconstitution of the immune system achieved by the infusion of haematopoietic stem cells from a healthy donor (Figure I.5). Historically, the first trials involving SCT in human patients were based on experimental work in murine and canine models and were attempted in the 1950's by Thomas *et al.* The results from mouse experimental models were the first to report the graft versus tumour (or graft versus leukaemia, GvL) effect when leukaemic cells were eradicated mice that received allogeneic (not genetically identical), but not syngeneic (genetically identical) bone marrow transplantation (Barnes *et al*, 1956). This suggested that a reaction originating from the donor haematopoietic stem cells could kill leukaemic cells in the host. However, the first experimental human SCTs were mostly unsuccessful and resulted in graft rejection in many cases of allogeneic transplants, but not in the case of a SCT transplant from a twin donor (Thomas *et al*, 1957; Thomas *et al*, 1959). In the latter case, haematological recovery was achieved, but the disease relapsed after a few months. The first case of a patient surviving after an allogeneic bone marrow transplantation was described in a leukaemia patient in whom chimerism, tolerance and anti-leukaemic effect were induced. Although the transplant was successful, the patient

later succumbed to the complications of graft versus host disease (GvHD). SCT settings have much improved since these early trials and involve several aspects of treatment and preventive strategies to better the patient's outcome by ameliorating the treatment while controlling the potential side effects and complications.

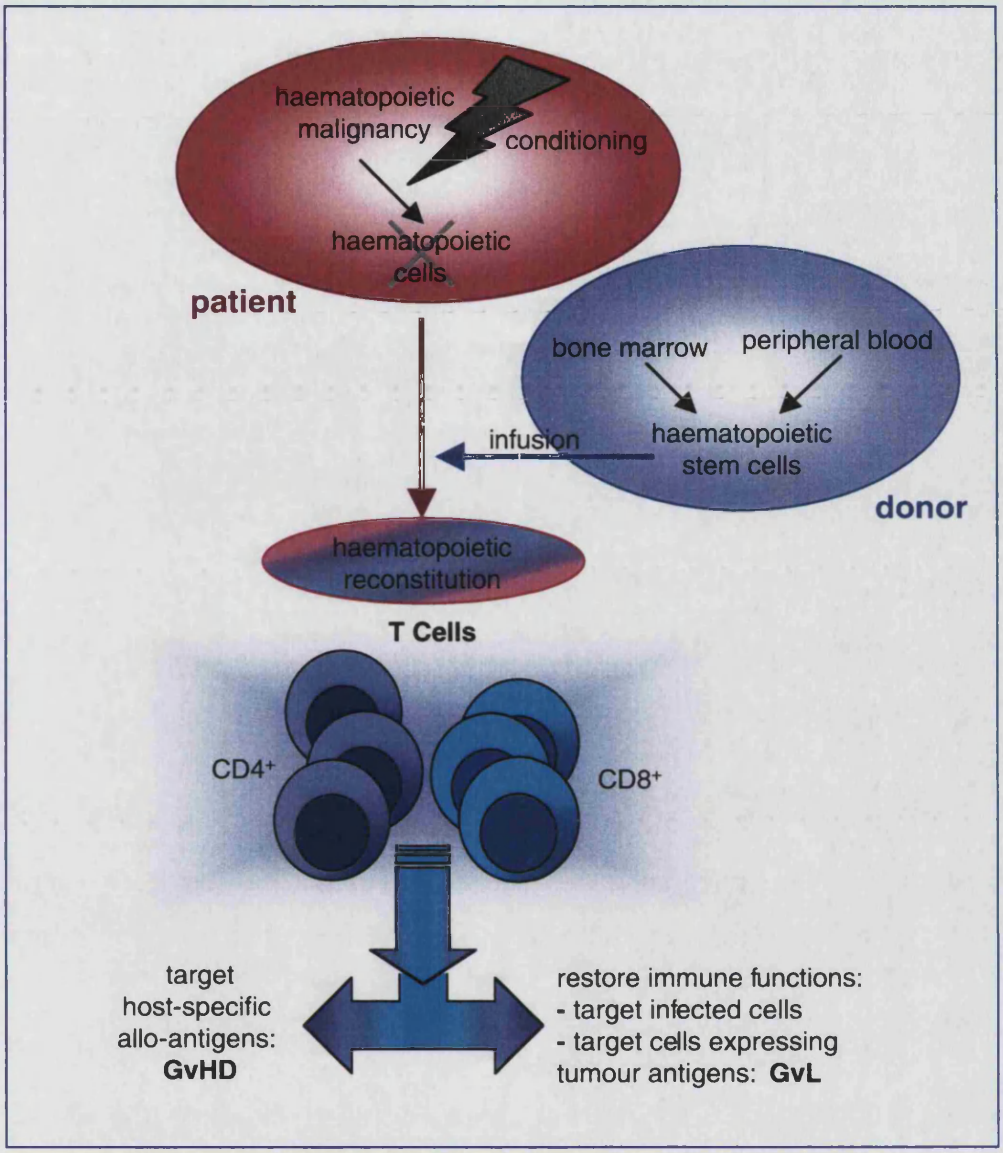

Figure I.5 Haematopoietic stem cell transplantation (HSCT)

HSCT is the only curative treatment option for haematological malignancies. This involves eliminating an individual's haematopoietic stem cells and all the cells that derive from them, including the hemopoietic, lymphoid, histiocytic and myeloid lineages. This is achieved by a process referred to as conditioning, which usually comprises both radiation and chemo therapies. The depleted stem cells are replaced by stem cells from another individual (or allo-SCT) which are infused in the patients' blood and will relocate to the bone marrow to reconstitute the haematopoietic system. A proportion of these cells will be $CD4^+$ or $CD8^+$ T lymphocytes directly present in the graft or reconstituted when haematopoiesis is re-established after transplant. These cell subsets mediate cellular adaptive immune responses. After HSCT, some of these responses can be beneficial and sought after in conducting the transplant procedure such as the response to pathogen infected cells and the graft versus leukaemia (GvL or anti-tumour effect); however, some are deleterious and can be life threatening such as graft versus host disease (GvHD, an immune response mediated by the grafted cells and directed towards host tissues).

Conditioning regimen

The conditioning regimens aim at ablating the patient's bone marrow and malignant cells, as well as at suppressing the host immune response so that the graft is not rejected. Total body irradiation (TBI) is often used because it has good immunosuppressive properties and acts against a wide variety of malignancies by causing damage to dividing cells and result in tumour cytoreduction. Irradiation treatment is usually applied in combination with chemotherapy with alkylating agents such as cyclophophamide, busulphan or melphalan and/or with antimetabolite agents such as methotrexate and mycophenolate mofetil, this mainly covers the agents used for the treatment of patients included in this PhD thesis. The common feature of the alkylating agents is that, upon cell entry, their alkyl groups bind to electrophilic sites in DNA and other biologically active molecules. As a result, the dominant bifunctional alkylation of DNA effect results in cross-links between DNA strands, which prevents subsequent DNA replication. By contrast, antimetabolite agents are analogues of biochemical substrates that lead to cell death (methotrexate) or are involved in the negative feedback of T cell activation (mycophenolate mofetil). Therefore both alkylating agents and antimetabolites can be combined to contribute to tumour cytoreduction and induce graft tolerance.

However, the most efficient way to obtain graft tolerance is to match the histocompatibility antigens of the patients with these of a stem cell donor.

Histocompatibility matching

In the absence of immunosuppressive drugs, the transplantation of haematopoietic stem cells from a healthy donor to a patient will result in an immune reaction that will lead to graft rejection. This reactivity results from differences in cell surface determinants such as MHC molecules or histocompatibility antigens (HLA for human cells). Non self MHC molecules are recognised by 1 to 10% of T cells. They are involved in the rejection of organs and tissues where very large numbers of T cells are specifically reactive to non self or alloreactive. This can occur through cross-reactivity of the TCR, which normally recognises foreign peptides displayed by self MHC molecules as described earlier. Two distinct mechanisms can be involved in such cross-reactive recognition: 1) peptide binding to non-self MHC may strongly interact with the TCR, which represents peptide dominant binding (Speir *et al*, 1998); 2) binding may occur directly between the TCR and distinctive features of a non self MHC molecule, which represents MHC dominant binding (Smith *et al*, 1997).

As only 35% of the patients who need SCT have HLA matched siblings, bone marrow donor registries have been set up, such as the Anthony Nolan Trust, and have compiled a resource of HLA typed volunteer donors willing to donate a fraction of their bone marrow or peripheral blood stem cells to patients who require stem cell transplants. Matching of patients and donors to achieve as complete as possible compatibility is achieved by matching the HLA types of the individuals concerned. The typing for major HLA molecules is usually performed at the molecular level, which includes typing for HLA class I genes: HLA-A, HLA-B and HLA-C; as well as for HLA class II genes: HLA-DR, HLA-DQ and HLA-DP prior to donor selection. However, even in allogeneic transplants where major HLA molecules have been fully matched, the donor infused lymphocytes can become sensitised to cell surface antigens that are expressed on patients' cells but are not a part of the MHC system. These are referred to as minor histocompatibility antigens and can mediate a graft versus host effect, one of the main complications after SCT.

Immune reconstitution

As described previously, the conditioning inherent to the SCT procedures leads to a stage of temporary but profound immunodeficiency. In particular, the early stage after SCT is characterised by a period of severe neutropenia when over 90% of the infections occurring in this phase are due to bacteria, to the reactivation of latent herpesviruses or to *Candida spp* invasion. At later stages after SCT, sibling and unrelated allotransplant recipients differ in their susceptibility to viral and fungal infections, as the intensity of the treatment they receive differs to prevent graft rejection and graft versus host disease (GvHD) as detailed below. At these stages, the pathogens that cause the most mortality are cytomegalovirus (CMV) and *Aspergillus fumigatus*, as both these are pathogens which are normally controlled by the host cellular immune response, this might suggest a deficiency in T cell reconstitution as well as neutropenia. The impaired T cell reconstitution after SCT is likely due in part to the limitations in the numbers of progenitor cell clones that were infused and survived and engrafted, as well as to alterations in the host microenvironment that disrupts normal T cell development. The factors that are known to contribute to alterations of the thymic environment as well as that of other lymphoid tissues include the age of the recipient, the toxicity of the conditioning regimen as well as the occurrence of GvHD (Mackall *et al*, 1997; Weinberg *et al*, 2001).

Complications after SCT

Treatment induced toxicity, a prolonged period of immunodeficiency and an extended recovery progress are at the origin of most post SCT complications. The major complications that are encountered include graft failure, GvHD, host defence defects and disease relapse. Graft failure resulting from the rejection of donor haematopoietic cells by host T cells that have survived the conditioning regimen can be prevented by *in vivo* T cell depletion of the host (Bunjes *et al*, 1987; Kernan *et al*, 1987) This process will be described in further detail below.

Graft versus host disease

The term GvHD describes the direction of the immunological damage caused by the introduction of immunologically competent cells in a immunoincompetent host. This phenomenon was recognised in SCT patients and also in immunodeficient children who were transplanted with allogeneic bone marrow (Lum *et al*, 1985). Its incidence and degree of severity correlates with the degree of HLA diversity between patient and donor (Lum *et al*, 1986), additionally, severe extended GvHD is associated with poor survival. It can be divided into two related syndromes, acute GvHD, which usually occurs within 30 days after SCT, and chronic GvHD, which occurs later than 100 days after SCT.

Acute GvHD is triggered by rapid and massive cytokine production or "cytokine storm" generated through T cell activation induced by genetic disparity between patient and donor, and as a direct consequence of the conditioning regimen toxicity (Ferrara *et al*, 1995). This perturbation of the cytokine network is considered to lead to a final common pathway involving macrophages and NK cells activated by T cell derived cytokines and resulting in organ damage involving the skin, liver and gastro-intestinal tract (reviewed in Antin and Ferrara, 1992 and Klingebiel and Schlegel, 1998). This response was characterised by the presence of $CD4^+$ T cells capable of producing IL2 and IFN-γ (or T helper 1), generally accompanied by a modest $CD8^+$ T lymphocyte infiltrate (Rus *et al*, 1995; Fowler *et al*, 1994; Szebeni *et al*, 1994). By contrast, chronic GvHD can either be interpreted as a late phase of acute GvHD or as a distinct disease with autoimmune features such as thymic atrophy, loss of thymal epithelial secretory function and lymphocyte depletion. Although multiple organs and organ systems may also be impaired, the skin and mucous membrane are the main targets of chronic GvHD. Furthermore, GvHD (both acute and chronic) is known to have deleterious effects on immune functions and cause profound lymphoid hypoplasia, B cell defects and damage

to the thymic stroma, which will result in impaired T cell development (Baker *et al*, 1997; Weinberg *et al*, 2001).

Prevention and treatment of GvHD

In early SCT studies, GvHD occurred in over half of the patients, even if they were HLA matched (Storb *et al*, 1977). The concept of GvHD prophylaxis resides in the fact that pharmacological immunosuppression induced at the time of SCT and for up to several months thereafter can prevent or blunt the initiation of T cell recognition and the proliferative response that is characteristic of GvHD. Significant improvements were made with the use of T cell depletion or inhibition of T cell activation for GvHD prophylaxis and treatment. This approach has successfully reduced the frequency and severity of GvHD. The agents that are used in the clinic to this effect include pharmacological drugs and immunological drugs (reviewed in Ho and Soiffer, 2001). The drugs used for GvHD prophylaxis are usually first administered at or near the time of transplant and include methotrexate, cyclosporin A and corticosteroids (Nash *et al*, 1992; Ramsay *et al*, 1982; Shorb *et al*, 1989; Chao *et al*, 1993).

Methotrexate is an analogue of aminopterin (folic acid antagonist) that acts by binding to dihydrofolate reductase (DHFR), subsequently preventing its ability to reduce oxidised folates to tetrahydrofolate, blocking further purine or thymidylate synthesis and therefore leading to cell death. Cyclosporin A represents a more targeted immunosuppressive function as it inhibits the production of IL2 without inducing cell death. It acts by binding to cyclophilin to form a complex that will inhibit the serine/threonine phosphatase activity of the calcium-activated calcineurin, which transmits signal from the TCR to the nucleus. Significant improvements in GvHD prevention have been made by combining methotrexate therapy with Cyclosporin A therapy (Storb *et al*, 1986; Ratanatharathorn *et al*, 1998). Similarly to methotrexate, corticosteroids have a wide activity range; they are a family of drugs that are related to steroids, which are naturally produced in the adrenal cortex, such as cortisone. They are powerful anti-inflammatory drugs that alter the transcription of many genes. The most widely used corticosteroid drugs prednisone and prednisolone are synthetic analogues of cortisol and can kill lymphocytes and especially developing thymocytes by inducing apoptotic cell death. They also affect cytokine production and cell trafficking. Cyclosporin A and corticosteroids are the main drugs that are used for the treatment of GvHD as well as prophylaxis, with the possibility of escalating the dose of steroids used to combat the symptoms.

The immunological drugs that are used for GvHD prophylaxis are usually administered prior to or at the time of transplant and include anti thymocyte globulin (ATG, also named anti lymphocyte or anti leucocyte globulin) and the more recently available Campath-1H. Although they are normally used to prevent GvHD by depleting alloreactive donor T cells, Campath-1H can also be used *in vivo* to prevent graft rejection by depleting patient T cells (Hale *et al*, 2000; Kottaridis *et al*, 2000). Both ATG and Campath-1H are immunoglobulins and act in a similar fashion by binding to their target on the cell surface and inducing their killing by phagocytosis or antibody dependent cytotoxicity. ATG is normally prepared by immunising horses with human lymphocytes, whereas Campath-1H is a recombinant humanised monoclonal antibody to CD52. CD52, a very short glycoprotein bearing a GPI anchor, is expressed at high levels ($5x10^5$ molecules per cell) on lymphocytes and monocytes, but not on plasma cells, platelets or erythrocytes. It is a very good target for complement mediated lysis and antibody mediated cellular cytotoxicity and CD52 monoclonal antibodies have proven to be very effective *in vivo* for lymphocyte depletion (Xia *et al*, 1993). Campath-1H monoclonal antibodies have been engineered with a human immunoglobulin framework and constant regions, but with the complementary determining regions of a rat monoclonal antibody to CD52 to reduce their immunogenicity (Jones *et al*, 1986; Riechmann *et al*, 1988).

The use of these drugs, and of these included in the conditioning regimen, which induce the suppression of the immune response allows the emergence of opportunistic pathogens to become manifest in these patients. As a result, the second most common complication after SCT is due to the β herpesvirus cytomegalovirus (CMV or human herpes virus 5, HHV-5).

Cytomegalovirus infection

In healthy individuals, CMV seroprevalence is widespread with up to 80% of individuals in the developed world having detectable levels of antibody to CMV by early adulthood (Nelson *et al*, 1997). Approximately 10% of primary infections in immunocompetent individuals are associated with a mononucleosis like syndrome, while the vast majority of primary infections remain clinically silent and mainly located to the salivary glands. At the end of the primary infection, CMV will have established lifelong latency mainly in peripheral blood mononuclear cells (monocytes) but can also infect and become latent in haematopoietic progenitor cells (Hahn *et al*, 1998; Mendelson *et al*, 1996; Sindre *et al*, 1996; Taylor-Wiedeman *et al*, 1994). Thus CMV is

able to reactivate when immune control fails and may affect haematopoietic reconstitution after SCT.

By contrast to the situation in healthy individuals, CMV infection is the major cause of interstitial pneumonitis in immunocompromised individuals such as patients after SCT or organ transplant, but also in individuals suffering from congenital immunodeficiencies or acquired immunodeficiency syndrome (AIDS) (Weiner *et al*, 1986; Ho, 1994; Yow and Demmler, 1992; Spector *et al*, 1996). CMV interstitial pneumonitis after SCT is related to CMV infection in 50% of incidences while the other cases include bacterial involvement or conditioning cytotoxicity (Inoue *et al*, 1990). CMV infection can also affect numerous other organs.

The most important risk factor predisposing to CMV infection is the seropositivity of the SCT recipient prior to transplant (Meyers *et al*, 1986; Miller *et al*, 1986), indicating that the majority of CMV infections after SCT are due to the reactivation of latent virus. Additionally, CMV related disease in SCT recipients is controlled primarily by a $CD8^+$ T cell response, however sustained viral control is also dependent on the presence of $CD4^+$ T cells (Li *et al*, 1994; Walter *et al*, 1995; Podlech *et al*, 1998). Indeed, the infection risk increases in recipients of unrelated donor allografts, as well as in recipients suffering from acute or chronic GvHD (Ochs *et al*, 1995; Hoyle and Goldman, 1994).

The understanding and assessment of the immune response to CMV and the application of adequate CMV prevention and treatment are therefore crucial to the improvement of patient care after SCT. All components of the virion can potentially be involved in immune targeting and are described below.

CMV structure and replication cycle

CMV viruses are species specific, and all the different human CMV strains are 95% homologous. The CMV virion has a complex structure composed of a large DNA core packed into an icosahedral capsid (Figure I.6, panel A). CMV has the largest genome of all herpesviruses that can be arranged into 4 different isomers of Long (L) and short (S) genomic strands that are separated by repeat sequences (Mocarski, 1996). The capsid is surrounded by an amorphous layer of tegument, which includes at least 20 different proteins. The most abundant proteins are pp65 (UL83) or lower matrix protein representing 95% of the tegument and pp150 (UL32) which is also the largest (Landini *et al*, 1987). The protein pp28 is also a tegument phosphoprotein involved in virus maturation but is not as abundant as pp65 or pp150. The tegument is enclosed in a cell

derived lipid membrane envelope containing virus encoded glycoproteins important for the attachment of the virus particle to the host cell. Once CMV has entered a cell by fusion of its outer membrane with the cellular membrane, the capsid then translocates to the nucleus where CMV DNA replicates. CMV DNA replicates in nuclear domains named viral replication centres where the first genes are transcribed; they are referred to as immediate early (IE, Figure I.6, panel B). The first translated CMV protein is IE-1 (UL123); it accumulates in the nucleus of infected cells where it can regulate viral gene expression, and constitutes an autoregulatory transactivator in primary or latent infected cells. This is followed by the transcription of early (E), then of late (L) genes. The newly replicated DNA is then inserted in preformed capsids in the nucleus. The particles then acquire some of their tegument proteins and primary envelope by budding from the nuclear membrane. The nucleocapsids are directed to the Golgi complex in the cytoplasm where they acquire additional tegument proteins and their mature envelope. The first stage of virion release is thought to occur through budding into Golgi derived vesicles that travel to the plasma membrane, where the virions are released by exocytosis (reviewed in Sanchez and Spector, 2002; Mettenleiter, 2002). The entire replication cycle of CMV is complete within 48 hr of infection in healthy individuals.

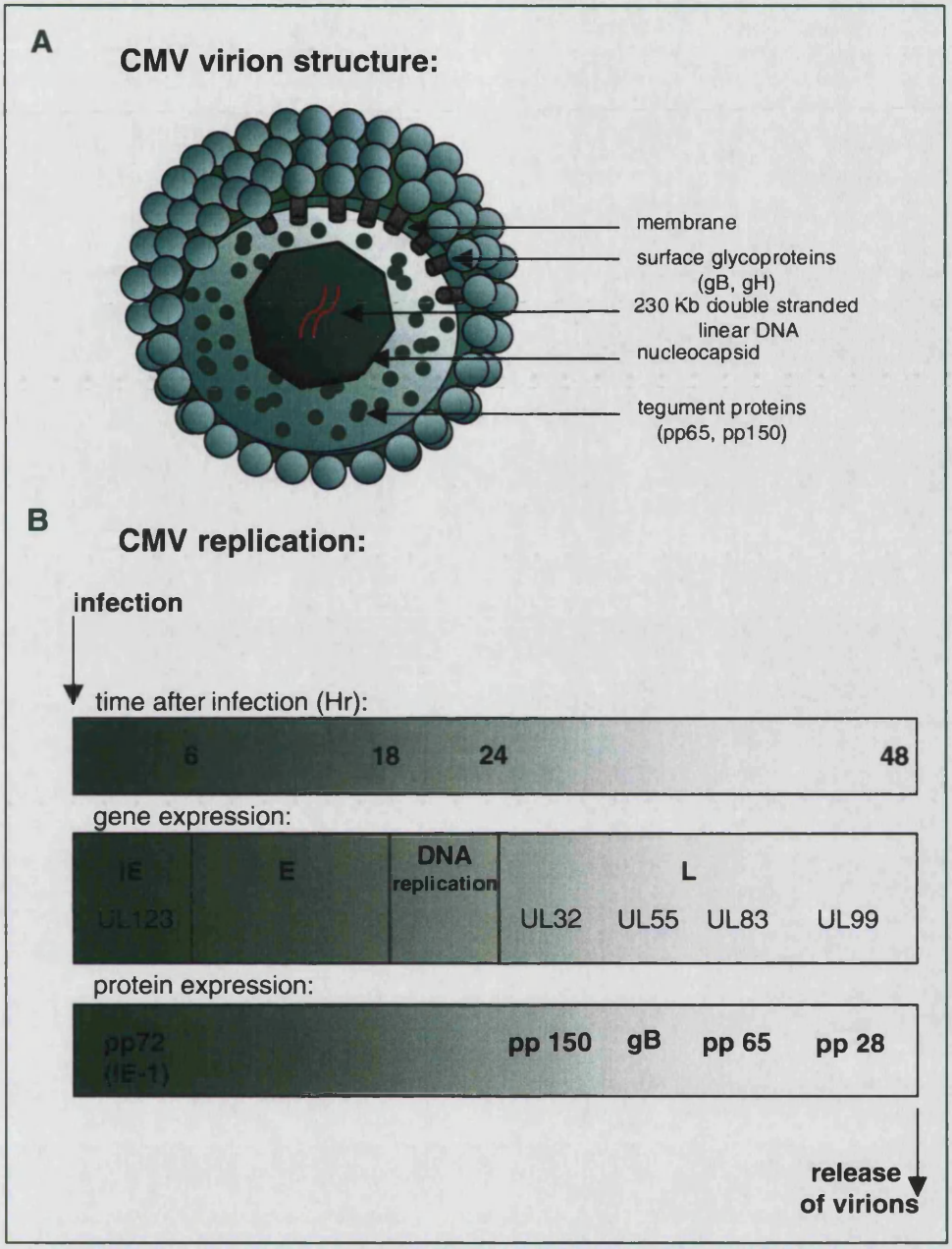

Figure I.6 Cytomegalovirus (CMV) virion structure and replication

Panel A: Schematic representation of CMV virion structure. The most abundant CMV components form part of the tegument component separating the phospholipid envelope (membrane) from the nucleocapsid enclosing the CMV genome. The tegument phosphoprotein 65 (pp65) is the most abundant CMV component accounting for 95% of tegument proteins. Panel B: CMV replication cycle. After attachment to the cell surface, and penetration of the virus in the target cell, full replication usually occurs within 48 hr from infection. The viral capsid and genome locate to the cell nucleus where viral genes are transcribed. They are referred to as upper long (UL) or upper short (US) genes depending on their situation on either side of a separating DNA repeat sequence in the CMV genome. Viral gene expression can be divided into 3 consecutive stages: 1) immediate early (IE) stage, which includes the expression of transcriptional transactivators that initiate and stimulates CMV replication such as IE-1 (also named phosphoprotein 72 or pp72); 2) early (E) stage, which includes the expression of proteins involved in DNA replication and metabolism; 3) late stage (L), which includes the expression of structural and packaging proteins.

Immune response to CMV

The adaptive immune response to productive CMV infection was shown to include both T and B cell responses. Additionally, the reconstitution of CMV specific immune responses in the context of SCT has been shown to be protective against CMV disease (Reusser *et al*, 1991; Li *et al*, 1994; Ljungman *et al*, 1993; Krause *et al*, 1997). Antibodies to the surface glycoprotein B (gB) can be detected in the blood of all CMV seropositive individuals, and this can be exploited to test if individuals have previously contracted CMV infection. The main targets for CTL reactivity were shown to be diverse depending on individuals and are mainly directed against pp65, IE-1 and gB, and their relative prevalence was assessed and established the immunodominance of pp65 (Wills *et al*, 1996; Boppana and Britt, 1996; Borysiewicz *et al*, 1988; Alp *et al*, 1991; Utz *et al*, 1992; Guylai *et al*, 2000). Further details about the CTL immune response to CMV will be discussed in Chapter III.

Diagnosis of CMV infection

The diagnosis of CMV infection is based on the detection of virus or viral particles in peripheral blood or biological samples. This used to be tested using viral culture techniques, which were very lengthy and somewhat unreliable. Diagnostic tools have now progressed significantly and involve the quantitative detection of CMV proteins such as pp65 by quantitative immunoassay (antigenemia) or the detection of CMV DNA by quantitative polymerase chain reaction (reviewed in Boeckh and Boivin, 1998).

CMV prevention and treatment

As described previously, the most powerful risk factor for the development of CMV infection after SCT is the seropositivity (CMV^+) of the recipient. Therefore the first means of CMV prevention after SCT to reduce the chances of CMV infection are to select a CMV seronegative (CMV^-) donor whenever possible and to use CMV^- blood products in early post transplant care. This guarantees that CMV infections in CMV^- recipients are extremely rare and if they did occur would result from a primary infection. CMV infection or reactivation has extremely serious consequences after SCT and can lead to interstitial pneumonitis, which once established is often non responsive to treatment and may result in fatal disease.

Efficient control of CMV replication can be achieved by prophylactic and treatment strategies with antiviral drugs such as Aciclovir, Valaciclovir, Ganciclovir

and Foscarnet. However, despite their good efficiency, none of these drugs are virus specific and are mainly virustatic so do not eliminate the virus entirely. Consequently, CMV infection will inevitably re-occur until adequate immune protection is recovered after SCT.

Aciclovir and Valaciclovir are mainly used for CMV prophylaxis after SCT and have moderate effect on CMV replication, but are most effective against herpes simplex virus (HSV) and varicella Zoster virus (VZV). They are acyclic analogues of deoxyguanosine; upon entry in the cell, they are phosphorylated to the monophosphate form by the CMV encoded UL97 protein kinase, then cellular kinases will phosphorylate these compounds to the di- then triphosphate forms (Matthews and Boehme, 1988; Littler *et al*, 1992; Sullivan *et al*, 1992). Active triphosphate drugs then act as a competitive inhibitor for the viral DNA polymerase. In the case of normal DNA elongation, the nucleoside hydroxyl group allows the binding for the next incoming nucleoside in the elongating DNA chain by the formation of a second phosphate ester bond. Aciclovir or Valaciclovir triphosphate lack the 3' hydroxyl group necessary to form this 3'-5' phosphodiester bond, their incorporation in DNA leads to termination (Perry *et al*, 1996; Smiley and Murray, 1996). Both these drugs have low levels of toxicity and in particular are not toxic to haematopoietic stem cells and do not show any immunosuppressive effect (Steele et al, 1980; McGuffin et al, 1980).

Ganciclovir (Gan) is also a deoxyguanosine analogue that contains a hydroxylmethyl group, which causes its improved antiviral activity compared to Aciclovir or Valaciclovir, but this also increases its cellular toxicity substantially. However Gan is very effective against a broad range of herpesviruses, and its mechanism of action is similar to that described previously. As with Aciclovir or Valaciclovir it is a competitive inhibitor of viral DNA polymerase, but the main difference resides in the fact that it is not an obligatory DNA chain terminator due to the presence of a 3'hydroxyl group. This fact is also the principal explanation for the high level of toxicity of Gan towards uninfected cells. Gan treatment needs to be monitored closely as Gan was shown to be mutagenic, carcinogenic, but particularly was also shown to inhibit the growth of haematopoietic stem cells as well as causing severe neutropenia. The latter two side effects of Gan treatment have very serious consequences in the SCT setting, and granulocyte colony stimulating factor (G-CSF) is used in combination with Gan therapy to increase the number of neutrophils (Salzberger *et al*, 1997; Randolph-Habecker *et al*, 2002). Additionally, Gan was shown to limit CMV specific immune reconstitution after SCT (Li *et al*, 1994).

By contrast with the drugs described previously, Foscarnet (Fos) or trisodium phosphonoformate is a non nucleoside molecule with a pyrophosphate group. It acts as a non competitive inhibitor of the viral polymerase without the requirement for cellular activation by cellular enzymes, by blocking pyrophosphate exchange from dinucleotide triphosphates (dNTPs) rather that incorporating in DNA. It is very efficient at suppressing CMV replication, but its major side effect is nephrotoxicity. Fos can be used as a second line therapy for patients who develop dose limiting neutropenia on Gan treatment, and is also often used in combination with Gan to prevent both viral replication and the emergence of resistant viral strains as both drugs have different antiviral activities. Dual resistance to both Gan and Fos occurs rarely.

These drugs can be used in different strategies after SCT: 1) prophylaxis, which involves treating CMV^+ patients from the time of SCT and continuously for the next 100 days either with low toxicity drugs or with Gan (Meyers *et al*, 1988; Prentice *et al*, 1997; Goodrich *et al*, 1991; Winston *et al*, 1993); 2) pre-emptive therapy administered at the time of detected antigenemia or viraemia with Gan or a combination of Gan with Fos (Boeckh *et al*, 1996; Hebart *et al*, 1996; Einsele *et al*, 1995). Both strategies have been shown to reduce the occurrence of CMV infection after SCT and to improve patient survival. However, the major toxicities associated with the use of these drugs gives rise to major concern that warrants the research for new drugs with lower toxicity and/or a different mode of action, and for non pharmacological treatment alternatives such as immunotherapy.

Association of CMV with GvHD

The association of CMV with GvHD has been well documented in patients after SCT (Miller *et al*, 1986; Einsele *et al*, 1988). This remains the case despite the continuous improvement in prophylaxis and treatment of both conditions. No causative effect has yet been demonstrated for the effect that both CMV infection and GvHD have on each other, and this will be the subject of further discussion in Chapter IV.

Cytomegalovirus immune evasion

CMV has devised multilevel strategies to influence its cellular host in order to avoid recognition. The first level of strategies used by CMV involve blocking the MHC class I processing and presentation pathway so that no viral peptides can be presented to T cells at the cell surface. Four separate CMV genes are responsible for this effect: 1) US3 is expressed at the beginning of CMV infection and impairs the transport of

MHC Class I molecules, inducing their accumulation in the ER (Ahn *et al*, 1996; Jones *et al*, 1996); 2) US2 and US11 are glycoproteins highly expressed at around 24 hours after infection, which misdirect MHC class I molecules by rapid translocation from the ER into the cytosol where they are degraded by proteasomes (Wiertz et al, 1996); 3) US6 is expressed at its highest levels from 48 to 96 hours after infection when the other genes interfering with the MHC Class I pathway are almost silent. It prevents peptide loading of MHC class I molecules by inhibiting TAP mediated peptide translocation into the ER (Lehner *et al*, 1997; Ahn *et al*, 1997; Hengel *et al*, 1997). As a consequence of the activity of these CMV genes, more than 90% of MHC class I molecules are absent from the cell surface of *in vitro* cultured fibroblasts at 24 to 48 Hr after infection. This constitutes the main known mechanisms for CTL evasion. However, this dramatic down-regulation of MHC class I by CMV would trigger recognition by natural killer (NK) cells, which react to the lack of self MHC molecules. Consequently, the second group of CMV immune evasion strategies is aimed at avoiding NK cell recognition and killing. This involves the production of MHC class I homologue molecules such as UL 18 and UL16, which provide a decoy function at the cell surface and engage killer inhibitory receptors (KIRs) on the surface of NK cells (Cosman *et al*, 2001). A second process also involves the up-regulation of the cell surface expression of the non-classical HLA-E molecule, which interacts with the NKG2-A receptor on the NK cell surface. The surface expression of HLA-E depends on the binding from conserved peptides derived from classical MHC class I molecules. However the same peptide is present in the leader sequence of the CMV glycoprotein UL40, thus inducing HLA-E expression and avoiding NK recognition. Finally, the last known strategy used by CMV to escape immune recognition involves the synthesis of an IL10 analogue, which inhibits macrophage activation, antigen presentation and $CD4^+$ T helper 1 polarisation (Rennick *et al*, 1997; Kotenko *et al*, 2000). The combined effect of these escape mechanisms makes CMV a successful pathogen and may in part contribute to the establishment of viral latency.

Disease relapse

In some instances when the conditioning regimen has not been sufficiently intense to eradicate the leukaemic clone, malignant proliferation reoccurs. There are then only two treatment options available to combat the returning malignancy, which are to perform a second SCT, or alternatively to attempt to rescue the patient by enhancing donor

derived immune responses against the leukaemia or graft versus leukaemia effect (GvL).

Graft versus leukaemia

The GvL effect was first described in a murine model of bone marrow transplantation, when mice that received an allogeneic bone marrow transplant showed eradication of the leukaemic cells, while these that received a syngeneic transplant did not (Barnes *et al*, 1956). Further evidence that alloreactive immune responses were mediated by T cells and were responsible for an anti-tumour effect came from their links with firstly the effects of GvHD, which was associated with a lesser risk of disease relapse, and secondly with T cell depletion, which was associated with a higher risk of relapse (Weiden *et al*,1981; Goldman *et al*, 1988). Therefore donor lymphocyte infusions (DLI) were introduced in an effort to enhance the anti-tumour effect and to restore disease remission. DLI was shown to be most effective in chronic myeloid leukaemia (CML) patients with impressive results of up to 75% of patients experiencing disease remission, but also caused significant GvHD associated with the procedure (Kolb *et al*, 1995; MacKinnon *et al*, 1995). The infused donor lymphocytes are thought to bind to surface antigens expressed on leukaemic cells in the context of MHC molecules and be specific for polymorphic minor histocompatibility antigens (or polymorphic self antigens presented to T cells), leukaemia associated antigens (or over-expressed leukaemia restricted self antigens presented to T cells) and leukaemia specific antigens. As CML is a well described disease resulting from a chromosomal translocation, it is constitute a good model disease for the study of the GvL effect.

Chronic myeloid leukaemia

The critical transformation events in CML affect immature $CD34^+$ progenitor cells and lead to uncontrolled proliferation. CML is characterised by the presence of a small abnormal chromosome named Philadelphia chromosome that results from the translocation between chromosome 9 and chromosome 22 (or t 9;22 (q34,q11)); (Nowell and Hungerford, 1960) , see Figure I.7.

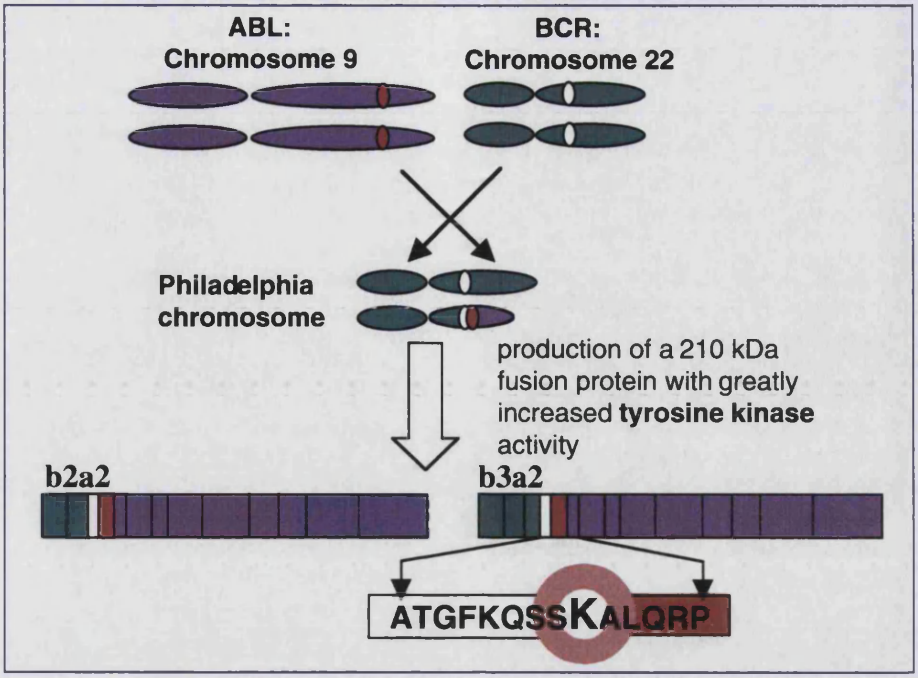

Figure I.7 Molecular etiology of Chronic Myeloid Leukaemia (CML): Philadelphia chromosome

Schematic representation of the Philadelphia chromosome resulting from the reciprocal translocation of the BCR gene situated on the long arm of chromosome 22 and displacing it to chromosome 9 (t 9;22), thus inserting within the ABL gene encoding for a tyrosine kinase. The majority of breakpoints in BCR and ABL are conserved and occur either after exon 2 or exon 3 of the BCR gene to join onto exon 2 of the ABL gene, creating the translocation types referred to as b2a2 and b3a2 respectively. The resulting chromosome 22 can be detected by karyotype analysis as one of the chromosome arms is shorter than the other. The BCR/ABL fusion gene is transcribed and translated as a functional protein characterised by the insertion of a new lysine (K) amino-acid at the intersection between the BCR and ABL gene segments.

Over 95% of the patients with CML have the Philadelphia chromosome and the leukaemic cells express a BCR/ABL chimeric protein (de Klein *et al*, 1982; Bartram *et al*, 1983). The translocation between chromosomes 9 and 22 juxtaposes variable sequences from the BCR gene (or breakpoint cluster region) on chromosome 22 to the majority of the c-ABL gene on chromosome 9. The breakpoints in the BCR gene occur either between BCR exons 2 (b2) and 3 (b3) or alternatively between exons 3 and 4. Therefore in the mature BCR/ABL mRNA, either b2 or b3 is spliced to exon 2 from c-ABL (a2), which results in two alternative chimeric p210 BCR/ABL proteins comprising either the b2a2 or the b3a2 junction with tyrosine kinase activity (see Figure I.7). The b3a2 resulting protein is further characterised by the insertion of a lysine residue (K) at the junction segment. This would therefore make it a good candidate for the investigation

leukaemic specific T cell responses. Comparisons between the p210 BCR/ABL protein and its normal homologue the p145 c-ABL kinase have indicated that this translocation modifies the intracellular location of the kinase from the nucleus to the cytoplasmic fraction (Oda *et al*, 1995; Yaish *et al*, 1988). Additionally, the fact that the BCR/ABL gene is under the control of the BCR gene promoter induces an up to 40 times over-expression of the protein in leukaemic cells compared to the expression level of c-ABL in normal cells. In experimental settings, the over-expression of c-ABL results in cell cycle arrest and induction of apoptosis whereas the expression of BCR/ABL promotes continuous cell cycle progression and diminution of sensitivity to apoptosis inducing agents (Melo, 1996; Wen *et al*, 1996). The expression of BCR/ABL is central to malignant transformation in CML, but the expression of p210 BCR/ABL appears to have a relatively subtle effect on the growth of haematopoietic progenitor cells and does not block their ability to differentiate (Fialkow *et al*, 1977). Consequently, CML manifests itself by an initial chronic phase of 2 to 5 years leading to a "blast crisis" phase, which resembles an acute leukaemia and is generally fatal.

Approximately 40% of CML patients express the b3a2 BCR/ABL protein, while another 40% express the b2a2 protein and the remaining 20% can express both transcripts. Both these transcripts are unique to BCR/ABL positive cells and therefore may constitute good candidates for the investigation of tumour specific T cell responses and the possible use of tumour specific immunotherapy for CML and/or relapse after SCT for CML.

Treatment of CML

The classical treatment for CML consists of conventional chemotherapy (described previously in the context of SCT conditioning regimen) to eradicate the malignant clone and induce cytogenetic remission, followed either by SCT or, for those patients who are not candidates for SCT, by long term treatment with IFN-α or Imatinib Mesylate (STI571, Gleevec or Glivec). Both these drugs can also be used to attempt to induce remission of disease relapse after SCT. IFN-α was until recently the standard treatment for newly diagnosed patients or for patients who were not candidates for SCT; it can induce remission in 70 to 80% of cases. The mechanism of action of IFN-α in CML remains unclear, but there is *in vitro* evidence of anti proliferative effects, of normalisation of adhesion and of improved cellular interaction of BCR/ABL positive cells. Additionally, there is some evidence for the promotion of NK and T cell cytotoxic activity by IFN-α, which is thought to contribute to the GvL effect. Imatinib Mesylate

was introduced recently as a selective inhibitor of the BCR/ABL tyrosine kinase. This synthetic molecule is an ATP mimetic that prevents the phosphorylation of the BCR/ABL substrate and can induce long term cytogenetic remission very efficiently with no major side effects (this is currently estimated at over two years after the induction of treatment, as this drug was only widely available for use in recent years), in contrast with IFN-α, which is not well tolerated (Druker *et al*, 1996). However, Imatinib Mesylate as a single agent does not appear to constitute a cure for CML, individuals do relapse and it is possible that overall it may eventually cease to be effective and disease will return, bringing the next treatment options of SCT and/or alternative immunotherapy.

Adoptive immunotherapy

Since the initial description of adoptive immunotherapy to describe haematopoietic SCT (Mathe *et al*, 1965), the term has evolved to include all immunological cells or molecules that can be used to that effect. Most of these strategies will require the prior definition of the antigen specific target (Boon *et al*, 1997). Many strategies are currently being tested and are aimed at enhancing anti-tumour or anti-viral immune responses. They include: 1) passive immunotherapy with monoclonal antibodies such as anti-idiotype monoclonal antibody for the treatment of B cell lymphoma (Miller *et al*, 1982) or cytokines such as IFN-γ, IFN-α and IL2 (described in further detail previously); 2) active immunotherapy with the enhancement of cellular cytotoxic responses, which can be achieved at different levels. The first possibility is to adoptively transfer antigen presenting cells (DCs) previously exposed to the antigen, or to transfer in vitro expanded antigen specific T lymphocytes (Szmania *et al*, 2000; Peggs *et al*, 2001). The second possibility consists in enhancing (or suppressing) the response of DCs or T cells to antigens. The strategies that have been used to this effect involve the optimisation of the interaction between the antigenic peptide, the antigen presenting cell and the T cell. For example, this could include the alteration of an antigenic peptide to increase its affinity to MHC molecules therefore enhancing T cell activation (Parkhurst *et al*, 1996). It could also involve the use or enhancement of co-stimulation, which was described previously and will be examined in Chapter V.

In the case of possible active immunotherapy approaches for CML, leukaemia reactive T cells can be selected and transferred into patients in order to induce cytogenetic remission (Falkenburg *et al*, 1999). As described previously, the targets for such responses in CML can be of different origin. Firstly, GvL can be induced by the

presence of minor histocompatibility antigen specific T cells (Horowitz *et al*, 1990). In some cases, antigens such as HA-1 and HA-2 show restricted expression to haematopoietic tissues and donors can show restricted cytotoxicity to cells expressing these minor antigens; however they are also linked to GvHD (Mutis *et al*, 1999). Therefore minor histocompatibility antigens may not constitute ideal targets for adoptive T cell immunotherapy. Other potential targets, which may have a reduced likelihood of inducing GvHD together with a GvL effect, include tumour associated antigens. These targets are proteins that are over-expressed in CML such as: 1) proteinase 3, a primary granule exclusively expressed in myeloid cells (Smit *et al*, 1997); 2) Wilm's tumour antigen, a zinc finger polypeptide that plays a key role in the pathogenesis of Wilm's tumour and is over-expressed in CML; 3) in contrast, telomerase, an enzyme which expression correlates with telomere lengthening is downregulated in accelerated phase CML, which is thought to contribute to genetic instability (Brummendorf *et al*, 2000; Engelhardt *et al*, 2000). Peptides derived from all these cited tumour associated proteins have been shown to be presented in the context of HLA molecules and to elicit antigen specific cytotoxic responses in vitro or to be associated with good prognosis (Molldrem *et al*, 1997; Molldrem *et al*, 2000; Minev *et al*, 2000;). Although these tumour reactive cells are associated with GvL activity, they are not solely restricted to the targeting of the tumour and may contribute to the GvHD effect. The only type of antigen that would generate a GvL response only would be restricted to a tumour specific antigen. In the case of CML, the b3a2 and the b2a2 proteins are good candidates for inducing anti-CML T cell responses and will be discussed in more detail in Chapter III.

The study of the physiological role of antigens specific immune responses in the context of immunodeficiency induced by the stem cell transplantation procedure has become possible with the labelling of antigen specific T cells.

Labelling antigen specific T cells

Direct labelling of antigen specific T cells has only become a possibility in recent years. This was due to the facts that unlike immunoglobulins, TCRs only exists as membrane bound molecules and have a very low affinity for their ligand (Braden *et al*, 1998; Garcia *et al*, 1996). Additionally, the labelling of antigen specific T cells relies on the prior knowledge of their MHC/peptide ligand combination, and in particular of the antigen specific peptide sequence. Without that knowledge or the possibility to use soluble MHC/peptide complexes to label antigen specific T cells because of the too low affinity for the TCR, the only methods available for the detection and quantification of antigen specific T cell responses relied on the use of transgenic T cells in mouse models of primary infection and on monitoring of the responses by detecting MHC/peptide restricted α or β chains at the T cell surface or by limiting dilution assay (MacDonald *et al*, 1993; Walker *et al*, 1995; Selin *et al*, 1996). The only barrier to labelling antigen specific T cells with soluble MHC molecules resided in their low affinity for the TCR. However the binding efficiency of a TCR to its ligand was shown to depend on both binding affinity, but also on binding avidity of the complexes, which included the relative density of the TCR at the cell surface (Schodin *et al*, 1996; Sykulev *et al*, 1998). The possibility of increasing MHC density to enhance the binding to TCRs and to extend the binding time was exploited by Altman *et al*, who resorted to multimerise MHC/peptide complexes by linking them to tetravalent streptavidin (Altman *et al*, 1996). This tetravalent complex or tetramer has enough avidity to form a stable bound with TCR molecules on the T cell surface, and labelled T cell can then be visualised by flow cytometry. The synthesis of such complexes will be described in further detail in Chapter II. This technique, in contrast with these described previously, allows the direct ex vivo visualisation and analysis of antigen specific T cells on a single cell basis. Alternative techniques to label antigen specific T cells have been developed since the discovery of MHC/peptide tetramers and include: 1) dimer MHC:immunoglobulin fusion proteins where the MHC molecule is fused to the variable region of the antibody heavy chain and the peptide of choice can be loaded onto the complexes prior to labelling. Similarly to MHC/peptide tetramer reagents, they possess increased avidity for the TCR, which allows their stable interaction (Fisher *et al*, 2002); 2) a cytokine capture method, which relies on prior labelling of the T cells with a cytokine capture antibody, IFN-γ for example, and stimulation with the specific antigen followed by secondary labelling of the antigen captured at the cell surface for fluorescent detection

or purification (Brosterhus *et al*, 1999). Although this technique allows the visualisation or purification of effector T cells only, it presents the advantage of being available to assess CD8 or CD4 restricted T cell responses.

Aims of this thesis

Haematological malignancies and their only available treatment SCT are at the origin of profound modifications of the immune system and its protective capabilities. Therefore an adequate recovery of immune functions is crucial to optimising patient survival after therapeutic intervention. Some of the most important factors implicated in decreased survival after SCT are due to disease relapse, viral infection or GvHD. As described in the previous chapter, it was shown that cytotoxic T lymphocytes play an important role in the elimination of virus infected cells, or cells expressing modified self-proteins (in some cases tumour cells).

Consequently, the aim of this PhD thesis was to develop an accurate method for the detection and quantification of the relevant antigen specific T cell responses to key components of the anti-tumour and anti-viral responses following SCT. The binding specificity of the T cell receptor for its HLA/peptide combination can be exploited to this end and required testing in order to confirm their potential usefulness for the immune response being studied.

This would allow one to use this information and these reagents in the investigation of a patient's progress throughout the course of their SCT, monitoring both their antigen specific immune reconstitution, and to correlate this information with the presence or absence of stimuli such as viral infections, as well as with the effects of various treatment procedures inherent in the SCT settings. This might be beneficial to the improvement of treatment protocols currently in place for elimination of tumour cells or virus-infected cells in this setting.

The accurate detection and monitoring of antigen specific T cell responses would in turn provide both targets and means of achieving efficient T cell adoptive transfer, by specific selection and/or activation of antigen specific T cells that might mediate the increase of antigen specific immune responses. This might provide new options for the treatment of tumours and/or infections in combination with previously established treatment strategies or on a sole basis in the context of haematological malignancies.

The findings from this PhD thesis and the techniques applied might be used in much wider areas where the detection, monitoring, and/or modulation of antigen specific immune responses would be useful. The materials and methods implicated in this project will be described in the following chapter.

Chapter II

Materials and Methods

Introduction

The materials and methods described in this chapter have been used to perform the work mentioned throughout all chapters.

Buffers and Solutions

All buffers and solutions mentioned in the following paragraphs have been prepared with chemicals purchased from BDH or Sigma. They were all filtered and autoclaved or filtered sterilised through 0.2 μm hydrophilic polypropylene filters (Gelman Laboratory) placed over re-usable bottle top filter units (Nalgene™).

Patient groups: recruitment and protocols

Recruitment and ethical approval

The patients recruited to participate in these projects were selected for the two distinct studies that were approved by local ethical approval authorities at the Royal Free Hospital (RFH), London and at the Liverpool Royal Infirmary where the studies were performed. All patients were undergoing treatment for haematological malignancies and had been typed for all major classical HLA antigens (HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP) at the allelic level and performed by molecular techniques if they were candidates for stem cell transplantation. HLA typing was performed at the serological level if the patients were on Imatinib Mesylate (STI 571 or Gleevec) therapy for chronic myeloid leukaemia (CML). The majority of all typing assays for both patient cohorts were performed at the Histocompatibility Laboratories at the Anthony Nolan Trust (ANT).

The patients testing positive for any of the HLA antigens relevant to our studies, HLA-A*0201 and HLA-A*0301 were selected as potential candidates. Additionally, in a second stage of the CML study, a few patients of unknown HLA type were also selected. This was done prospectively as the study was extended to cover other HLA

types of interest which were being studied by a fellow student, Sylvie Rusakiewicz, and also to collect samples that could act as irrelevant HLA type controls for our experiments. On receipt of the first blood sample, these patients were typed serologically for their HLA phenotype so that they could be categorised to the test or control groups.

A member of the medical team from the treating hospital approached each patient and informed him/her of the objective of the study they could enter. Each patient was given an information sheet summarising the objectives (see Appendix) and the protocol of the study that they could choose to enter into. Subsequently, the patients who decided to enter the study were asked to sign a consent form (see Appendix).

Blood collection protocols

In parallel with blood being taken for other routine tests required to assess patient progress at clinic visits, an additional peripheral sample of 20 ml was drawn; therefore participating in these studies did not require any additional venupuncture for the volunteers. The blood samples obtained from Crowley Ward at the RFH were drawn into tubes containing 20 µl of Heparin (Monoparin (1000 U/ml), CP Pharmaceuticals) to prevent coagulation and were collected on the same day so that they could be processed as promptly as possible.

The blood samples obtained from the Liverpool Royal Infirmary were drawn into tubes containing 20 ml of transport medium to which an equal volume of blood was added. Briefly, sterile Falcon tubes (50 ml, BD) were filled with 20 ml of transport medium composed of sterile filtered RPMI 1640 cell culture medium supplemented with tri-sodium citrate (at a final concentration of 3.3% (w/v)) and β-mercaptoethanol (at a final concentration of 5 µM). They were sent to Dr Richard Clark, our collaborator at the Liverpool Royal Infirmary, and were used to collect and dispatch peripheral blood samples that were sent by overnight mail delivery service so that they could be processed on the following day at the Anthony Nolan Research Institute (ANRI).

Control blood samples

Blood samples from healthy laboratory personnel from the ANRI and the RFH department of haematology were obtained. All samples were typed for the major HLA antigens at the allelic level by molecular techniques at the ANRI. Fourteen individuals were selected to provide control samples for the CMV study and had the following characteristics: 8 were HLA-A*0201 positive and CMV seropositive; 5 were HLA-

A*0201 positive and CMV seronegative; and one was HLA-A*0201 negative (HLA-A*30/HLA-A*31) and CMV seropositive.

Patients' characteristics

CMV study

CMV serostatus

Previous human Cytomegalovirus (CMV) infection is characterised by the presence of specific immunoglobulins (Ig) in the serum. A recent infection can be confirmed by the detection of both IgM and IgG subtypes; an older infection is characterised by the presence of the IgG subtype only.

All patients recruited into the CMV study and their respective donors were assessed for a previous CMV infection (Table II.1). Also, all of our healthy volunteers recruited in the laboratory were also tested for their CMV serostatus.

This was performed in the Histocompatibility Laboratories at the ANT, using the Bioelisa CMV IgG kit (Biokit) to test heat inactivated serum (this is necessary to avoid false positive results). Briefly, the test consisted of adding diluted sera specimen to inactivated CMV antigen coated on a 96-well plate (U 96 Maxisorb immunoplate, Nunc). If there were antibodies present in the sample tested, they bound to the antigen coated wells and, after washing, remained in the well while the rest of the sample was discarded. An enzyme labelled antibody to human IgG (rabbit anti-human IgG coupled to peroxidase) was then added. This bound to any CMV antigen/CMV IgG complex formed in the previous step. After another washing step to eliminate any excess or unbound material, the enzyme substrate solution (citrate-acetate buffer containing hydrogen peroxide) containing a chromogen (3,3'-5,5'-Tetramethylbenzidine dissolved in dimethylsulphoxide) was added. A blue colour developed if the sample tested contains CMV IgG (0.25-2.5 IU/ml). The blue colour was converted to yellow upon addition of sulphuric acid, which stopped the reaction and the optical density of the sample was measured at 450 nm. The intensity of the colour was proportional to the amount of CMV IgG in the tested samples and gives a qualitative result, as compared with negative, low positive and highly positive controls.

Sample collection and consultation of clinical data

PBMC samples were obtained from Crowley Ward at the RFH, and routine haematology clinical data was consulted with the help of research nurse Sarah Grace.

This included general patient information, cell counts determined at the same time as the samples that were analysed for our studies so that the absolute number of HLA-peptide tetramer cells could be calculated after staining. The qualitative cytomegalovirus polymerase chain reaction results from the RFH Department of Virology were also obtained.

Patients' characteristics

Twenty-five HLA-A*0201 positive patients treated for haematological malignancies by bone marrow or peripheral blood SCT in 1999 and 2001 at the RFH were enrolled in the study after they gave informed consent. Patient details as well as the patient and donor CMV serology are summarised in Table II.1. Conditioning prior to transplant for those 25 patients consisted of total body irradiation (n=18), cyclophosphamide (n=21), melphalan (n=4), busulphan (n=1) with additional fludarabine (n=15) and *in vivo* Campath-1H (10 or 20 mg for 5 days, n=14). Twelve patients received a T cell depleted graft (10 or 20 mg of Campath-1H with the graft also referred to as "in the bag") and no or low post-transplant immunosuppression. Patient 8 received a graft prepared by CD34 selection (an indirect form of T cell depletion). Thirteen patients received a non-T cell depleted graft and post-transplant immunosuppression with Cyclosporin A and a short course of Methotrexate. The patients were monitored weekly from engraftment if they had sufficient cell counts until discharged from hospital and at each visit to the clinic thereafter. From the start of conditioning until engraftment, patients received high dose intravenous Aciclovir (800 mg, 4 times a day), to suppress CMV (as well as Varicella Zoster and Herpes Simplex viruses). Aciclovir or Valcyclovir were also used preventively between courses of intravenous Ganciclovir (5 mg/kg of body weight twice a day) ± Foscarnet (90 mg/kg of body weight) antiviral therapy and continued until at least five months post-transplant. CMV DNAemia was assessed by routine qualitative PCR performed at the RFH Department of Virology (Royal Free Hospital). Treatment with Ganciclovir ± Foscarnet was initiated after two consecutive positive tests for ten days or until the CMV PCR amplification became negative. Simultaneously to Ganciclovir, patients were given GCSF to counteract the myelo-suppressive effect of Ganciclovir. Patients were followed closely for acute or chronic graft versus host disease (GvHD) and treated accordingly with Methylprednisolone (the dose and length of treatment varied depending on the severity of the symptoms and the response to treatment) and or horse anti-leucocyte globulin (ALG).

Patient	Disease[a]	Sex	Age, years	Patient/Donor CMV serology[b]	Stem cell source[c]	Conditioning regimen[d]	Campath-1H *in vivo*/in graft	GvHD prophylaxis[e]	Follow-up, days
1	AML	M	39	+/ -	SIB PB (F)	Cyclo and TBI	No/ Yes	None	495
2	ALL	F	40	+/+	SIB PB	Melph and TBI	None	MTX and CsA	188-603
3	AML	M	32	+/+	MM UD BM (F)	Flu, Cyclo and TBI	Yes/Yes	CsA	102-419
4	AML	M	54	+/+	SIB PB	Cyclo and TBI	None	MTX and CsA	447
5	AA	M	30	+/+	SIB PB	Flu and Cyclo	Yes/No	MTX and CsA	531
6	NHL	M	48	+/+	SIB PB	Cyclo and TBI	None	MTX and CsA	553
7	BJ M	F	49	+/+	SIB PB (MM)	Cyclo and TBI	No/ Yes	None	221
8	AML	M	35	-/-	SIB PB	Cyclo and TBI	None	None	793
9	ALL	F	18	-/-	UD BM	Flu, Cyclo and TBI	Yes/Yes	None	682
10	AA	M	21	-/-	SIB PB	Flu and Cyclo	Yes/No	Myco and MTX	506
11	AML	F	15	-/-	SIB BM (MM)	Flu, Cyclo and TBI	Yes/No	MTX and CsA	376
12	NHL	F	54	+/+	SIB PB	Melph and Flu	Yes/No	MTX	111
13	CML	M	33	+/+	SIB PB	Cyclo and TBI	None	MTX and CsA	66
14	CML	F	25	-/-	UD BM (MM)	Flu, Cyclo and TBI	Yes/Yes	CsA	125
15	WAS	M	18	+/ -	UD PB	Melph and Flu	Yes/No	MTX and CsA	149
16	ALL	M	11	+/+	SIB PB	Cyclo and TBI	None	MTX and CsA	131
17	CML	M	25	+/+	UD BM	Flu and Cyclo	Yes/Yes	None	244
18	MDS	M	23	+/+	SIB BM (F)	Flu, Bus and Cyclo	Yes/No	MTX and CsA	97
19	AML	M	40	+/ -	UD BM (MM,F)	Flu, Cyclo and TBI	Yes/Yes	CsA	227
20	AML	F	31	+/+	UD PB (M)	Flu, Cyclo and TBI	Yes/Yes	CsA	242
21	CML	F	41	+/ -	SIB PB	Flu, Cyclo and TBI	No/ Yes	None	39
22	AML	M	12	- /+	UD BM	Flu, Cyclo and TBI	Yes/Yes	CsA	180
23	CLL	M	51	+/+	UD PB (F)	Flu and Melph	None	MTX	119
24	AML	M	18	+/ -	UD PB (MM)	Flu, Cyclo and TBI	Yes/Yes	CsA	134
25	AML	M	37	+/ -	SIB PB	Flu, Cyclo and TBI	No/ Yes	None	93

Table II.1 CMV study: Patient's characteristics

This table summarises the characteristics of all 25 HLA-A*02 positive patients recruited to the cytomegalovirus study.

[a] ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BJM, Bence Jones Myeloma; NHL, non-Hodgkin's lymphoma; AA, aplastic anaemia; CML, chronic myeloid leukaemia; WAS, Wiskott-Aldrich syndrome; MDS, myelodysplastic syndrome; CLL, chronic lymphocytic leukaemia.

[b] +, cytomegalovirus serology positive; -, cytomegalovirus serology negative. [c] SIB, sibling; UD, unrelated donor; PB, peripheral blood; BM, bone marrow; MM, HLA mismatched; (F), sex mismatched transplant of a male patient with a female donor. [d] Cyclo, cyclophosphamide; Flu, fludarabine; Melph, Melphalan; Bus, busulphan; TBI, total body irradiation. [e] CsA, cyclosporin A; GvHD, graft-versus-host disease; MTX, methotrexate; Myco, mycophenalate. Patients could be segregated into different groups depending on their CMV serostatus: seronegative patient and donor; seropositive patient and donor, blue; seropositive patient and seronegative donor, yellow; seronegative patient and seropositive donor, pink. They could also be separated into different levels of Campath-1H T cell depletion (TCD): no TCD; in vivo TCD: red; TCD graft: green. The highest level of T cell depletion being highlighted with both red and green.

CML study

CML transcript type

Transcript specific polymerase chain reaction (PCR) was performed after extraction of mRNA from PBMC samples at the treating hospital and was verified prior to sample collection. The majority of the patients in this study were chosen for their possession of the BCR/ABL b3a2 transcript, which was the fusion protein transcript targeted in our study.

Patients' characteristics

The clinical details of the patients and controls included in the CML tetramer study are summarised in Table II.2, together with HLA assignments and BCR/ABL transcript type. The first four patients entered in the tetramer staining study (patients 26 to 29) expressed the b3a2 transcript. The controls were one BCR/ABL negative AML patient (patient 30) and a patient with severe aplastic anaemia (Patient 5, as a control for tetramer staining, see also Table II.1), three patients were positive for HLA-A*0301 and two patients were negative for HLA-A*0301. The remaining patients, patients 31 to 37 were also tested by tetramer staining, whereas patients 38 to 45 (as well as control patient 6, see Table II.1), were enrolled in the second phase of the study, where PBMC samples were collected and cryopreserved and the HLA type was only determined retrospectively. They were used in specific stimulation experiments described in Chapter V.

Patient	HLA type	Disease[b]	Transcript type	Treatment
26	A2/A3	CML	b3a2	SCT[d]
27	A3/A29	CML	b3a2	SCT
28	A2/A3	CML	b3a2	SCT
29	A1/A31	CML	b3a2	SCT
30	A2/A2	AML	NA[c]	SCT
5	A2/A30	AA	NA	SCT
31	A1/A3	CML	b2a2	SCT
32	A1/A3	CML	b3a2	autograft + IFNα[e]
33	A3/A3	CML	b3a2	SCT + DLI[f]
34	A2/A68	CML	b3a2	SCT
35	A3/A11	CML	b3a2	SCT
36	A3/A29	CML	b3a2	SCT + IFNα
37	A1/A2	CML	not available	SCT + DLI
38	A2/A3	CML	b3a2	IM[g]
39	A3/A3	CML	b3a2	SCT
40	A3/Un	CML	b3a2	SCT
41	A2/A3	CML	b3a2	IM
42	A2/A3	CML	b3a2	IM
43	A3/Un	CML	b3a2	SCT
44	A3/A11	CML	b3a2	IM
45	A1/A3	CML	b3a2	IM
6	A2/A3	NHL	NA	SCT

Table II.2 CML patient characteristics

This table summarises the characteristics of all patients entered in the tetramer CML study. Serological HLA types are reported, as well as the type of disease the patients were treated for and the BCR/ABL transcript type of their tumour.
[a] Cytomegalovirus; [b] CML: chronic myeloid leukaemia, AML: acute myeloid leukaemia, AA, aplastic anaemia, NHL, non-Hodgkin lymphoma; [c] NA, not applicable; [d] stem cell transplantation; [e] interferon-α; [f] donor leucocyte infusion; [g] Imatinib Mesylate (Gleevec or STI 571). A line separates the group of patients studied in Chapter III from the group of patients studied in Chapter V.

Cell separation and cell culture

Peripheral Blood Mononuclear Cell (PBMC) separation

PBMCs were isolated from whole blood by density gradient centrifugation. This consisted of layering a volume (typically 10 ml) of blood over an equal volume of Lymphoprep™ (Axis-Shield) reagent in a sterile Universal tube (Bibby Sterilin), followed by centrifugation at 1,000 g for 25 min at room temperature (RT). The centrifugation was terminated without braking so as not to disturb the interface cellular layer. PBMCs were then recovered from the interface layer and transferred to a fresh 50 ml Falcon tube (Becton Dickinson) containing a small volume of RPMI 1640 (with L-Glutamine, Bio Whittaker) cell culture medium. The tubes were then filled with RPMI

1640 medium to wash the cells free from remaining cell separation reagent, the tubes were then centrifuged at 680 g at RT for 10 min. This last step was repeated, and the cell pellets were resuspended in 1 ml of RPMI 1640, counted, and split into two fractions: one of approximately $2x10^6$ cells to be stained directly with HLA tetramers and the rest to be cryopreserved for later analysis.

Cell enumeration and viability

Cells were mixed to obtain a homogenous suspension before a 10 µl aliquot was taken. This was mixed with an identical volume of 0.4% sterile filtered Trypan Blue dye solution (Trypan Blue powder, BDH) in Phosphate Buffer Saline (PBS, 1.7 µM KH_2PO_4, 50 mM Na_2HPO_4, 150 mM NaCl, pH7.4) to stain for dead cells. The cell suspension was then placed in a counting chamber (Neubaur Chamber, Weber). The number of live (translucent) and the number of dead cells (staining blue) were counted using a phase contrast microscope (DMLB, Leica) to estimate the concentration and the viability of the cells in culture.

Cell Cryopreservation

Cells to be cryopreserved were transferred to a 50 ml Falcon tube (BD), this was then filled with RPMI 1640 (with L-Glutamine, Bio Whittaker) warmed at 37°C before centrifugation at 680 g for 10 min at RT. The supernatant was discarded and the cells were immediately re-suspended in RPMI supplemented with 10% heat inactivated (1 hr at 55°C) sterile filtered Foetal Calf Serum (FCS, Serum Supreme from Bio-Whittaker) and 15% Dimethylsulphoxide (DMSO). Aliquots of 1 ml at a maximum concentration of $10x10^6$ cells per ml were then transferred to 1.5 ml cryovials (Cryotube Vials, Nunc) and immediately placed in a polystyrene box at -80°C for 24 hrs. The next day, vials were placed in a liquid nitrogen tank for long term storage.

All primary human leucocyte samples that were cryopreserved were put back in culture for at least 12 hr prior to setting up any assays or stainings to allow for recovery of cell surface molecules and cellular function.

Cell culture

Cells were cultured at 37°C in a humidified incubator (IG 150, Jouan) with 5% CO_2. If the cells were used following a period of cryopreservation, they were first transferred to a 50 ml Falcon (BD) tube, and were washed in 50 ml warmed (37°C) RPMI 1640 (with L-Glutamine, Bio Whittaker) and centrifuged at 680 g for 10 min at RT. This step was

repeated to ensure removal of DMSO, then the cells were re-suspended in warm (37°C) RPMI 1640 medium supplemented with 10% FCS or human AB serum and placed in either sterile tissue culture dish or flask).

Primary Leucocyte culture

Freshly isolated human primary PBMCs were cultured in RPMI 1640 (with L-Glutamine, Bio Whittaker) supplemented with 1% (v/v) Penicillin/Streptomycin (respectively 10,000 units/ml and 10,000 µg/ml, Bio-Whittaker) to prevent bacterial contamination. The medium was also enriched with 10% heat inactivated sterile filtered (1 hr at 55°C) human AB serum (Bio-Whittaker). Cells in suspension were dispensed at 0.5 or $2x10^6$ cells per ml of supplemented medium in 24 well plates (Falcon, BD). Interleukin (IL) 7 and/or IL 15 were also added typically every 3 to 4 days or when culture conditions dictated to ensure the survival and growth of lymphocytes. As soon as the cultures showed signs of medium exhaustion as shown by a change of pH and indicated by a change in the cell culture medium colour, half of the medium was renewed.

CMV specific T cell line culture

Following CMV tetramer staining and single cell sorting, Mirjam Heemskerk, a collaborator at the Leiden University Medical Center, raised a CMV tetramer specific CD8 T cell line. This line was stimulated with antigen specific peptide and was grown in Dulbecco's Modified Eagles Medium DMEM) supplemented with penicillin/streptomycin and 10% human AB serum (both from Bio-Whittaker) before use in assays.

Immortal cell culture: T2

Unlike primary cells, the T2 cell line results from a fusion between an EBV transformed B cell line and a T lymphoma cell line. It is an immortalised cell line selected for a lack of HLA class I expression. T2 cells only require a basic cell culture medium to proliferate *in vitro*. For our experiments, they were grown in RPMI 1640 (with L-Glutamine, Bio Whittaker) supplemented with Penicillin and Streptomycin to prevent bacterial infections and with 10% heat inactivated FCS. Typically, they were cultured in flasks (15 ml, BD) and grown at a concentration of 0.3 to $0.7x10^6$ cells/ml of medium; medium was renewed when it showed signs of exhaustion. On the day preceding an experiment, T2 cells were counted and cultured at 0.5 to $0.6x10^6$ cells/ml of fresh medium to ensure maximum cell viability.

Some cells ($2x10^6$) were cryopreserved to replace cell stocks and as a backup vial that could be accessed if there were any results that needed confirmation.

Peptide pulsing of T2 cells

The T2 cell line is HLA-A*0201 positive but TAP (Transporter associated with Antigen Processing) deficient; the endogenous HLA-A*0201 molecules can be stabilised by using the leader peptide from HLA-A2 and consequently will present folded HLA-A*0201 molecules at the T2 cell surface. However these tend to be somewhat unstable and only small amounts of refolded HLA/peptide complexes can be detected at the cell surface. It is possible to compete out any remaining leader peptide and to stabilise empty molecules at the cell surface by pulsing the cells with a higher affinity peptide of choice.

Typically for peptide pulsing, T2 cells were cultured at $1x10^6$ cells/ml of medium and were incubated with 10 mg/ml of CMV specific peptide for 3 to 4 hours. The peptide excess was then washed off: the cells were transferred to a Falcon tube (BD) that was then filled with RPMI 1640 (with L-Glutamine, Bio-Whittaker) warmed at 37°C, and the tube was centrifuged at 680 g for 10 min at RT. The supernatant containing the excess peptide was discarded and the cells were resuspended in fresh supplemented culture medium, ready for use in assays.

Cell labelling

PKH-67 and PKH-26 membrane dyes

The long aliphatic tailed PKH-67 (MINI-67 kit, Sigma) and PKH-26 (MINI-26 kit, Sigma) dyes are composed of respectively a green and a red fluorescent molecule that can incorporate into the cell membrane lipid layer by selective partitioning.

An aliquot of $2x10^7$ T2 cells was taken and the cells were washed once with RPMI 1640 (with L-Glutamine, Bio-Whittaker) without serum. The cells were centrifuged at 680 g for 5 min into a loose pellet. The supernatant was carefully aspirated, leaving no more than 25 µl at the bottom of the tube and the pellet was resuspended by gentle tapping. First, the cells were resuspended and dyed according to the manufacturer's instructions. The labelling reaction was then stopped by adding an equal volume of sterile filtered heat inactivated FCS (Serum Supreme, Bio-Whittaker), followed by the addition of an equal volume of medium. The cells were centrifuged at 680 g for 10 min at RT to remove the excess staining solution, then washed with supplemented medium before repeating the centrifugation cycle. The labelled cells were

then resuspended at $1x10^6$/ml of medium and their fluorescence checked using a fluorescence microscope (DMLB, Leica) before use in assays (PKH 67 labelling, Figure II.1). This was used to label the T2 cell population so that it could be distinguished from PBMCs by flow cytometry or fluorescence microscopy in killing assays using the T2 cells as targets.

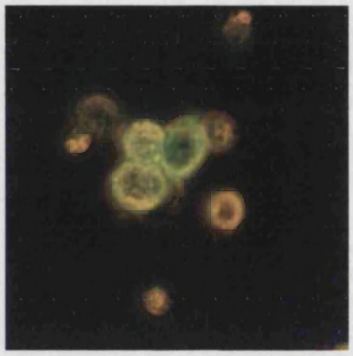

Figure II.1 PKH 67 labelling of T2 cells

Cell membrane labelling of T2 cells with the green fluorescent compound PKH 67 was checked by fluorescence microscopy with a DMLB Leica microscope at a forty times magnification. The photograph was taken using the WILD MPS 52 Photoautomat (Leica).

Cytokines and polyclonal stimulation

The following cytokines and stimulator were periodically added to primary leucocyte cell cultures:

Interleukin 2

Recombinant human IL 2 (R&D Systems) was used to promote CD8 T cell proliferation after antigen specific stimulation. It was used three days after antigenic stimulation at a concentration varying between 10 and 50 U/ml depending on the experimental setting.

Interleukin 7

Recombinant human IL 7 (R&D Systems) was used to promote lymphocyte survival and proliferation and was added at the beginning of the culture, three days after antigenic stimulation and at each medium change thereafter at a concentration of 10 ng/ml of culture medium.

Interleukin 12

Recombinant human IL 12 (R&D Systems) was used to promote naïve T cell priming and proliferation. It was added simultaneously to the antigenic stimulation on the first day of culture. It was used at a concentration of 50 U/ml of culture medium.

Interleukin 15

Recombinant human IL 15 (First Link Ltd) was used as a potent lymphoid cell growth factor. It binds to the IL15 receptor (composed of identical β and γ chain sub-units as the IL2 receptor, but with a distinct α chain sub-unit) on the T cell surface and induces proliferation. It was used three days after antigenic stimulation and at each medium change thereafter at a concentration of 10 ng per ml of culture medium.

Polyclonal stimulation

Purified Phytohemagglutinin (PHA, Abbot Murex) is a non-specific mitogenic stimulus that can induce polyclonal T cell mitosis, in a similar way to poly-antigenic stimulation. It was used in tissue culture experiments and ELIspot (Enzyme Linked Immuno-spot) assays to act as strong promoter of T cell proliferation and activation and to act as a positive control. It was used at a concentration of 1 μg/ml of culture medium in parallel to antigen stimulation tests and negative controls (no stimulation).

Staining Solution

Freshly isolated PBMCs and cultured cells were tested for T cell receptor specificity by staining for with HLA-peptide tetramers and/or for cell surface marker expression by staining with fluorescently labelled antibodies. These staining reactions were performed in sterile filtered RPMI 1640 medium (with L-Glutamine, Bio-Whittaker) containing 10% of heat inactivated FCS (Serum Supreme, Bio-Whittaker) and 5 mM Sodium Azide (NaN_3, BDH) to prevent internalisation of the receptor/antibody complexes.

Fixative solution

Stained cells were resuspended in a small volume (200 μl) of fixative solution composed of RPMI 1640 (with L-Glutamine, Bio-Whittaker) and 1% of paraformaldehyde (BDH). Although this killed the cells, it ensured that the bound antibody and tetrameric molecules would not dissociate from their ligand molecule on the cell surface. Samples were analysed by flow cytometry within 24 hours.

Molecular biology and bacterial cloning

Purification of Ribonucleic acid (RNA) from cells

Cryopreserved PBMCs from a selected HLA-A*0201 healthy individual and a selected HLA-A*0301 healthy individual were cultured briefly overnight before they were used for RNA extraction. This was done using the RNeasy mini Kit (Qiagen), using Rnase free materials on a cleaned and RNase free bench (RNase Away, Molecular Bio Products). The cells ($5x10^6$) were first pelleted and resuspended in RNA Stabilisation Reagent, then they were lysed, homogenised, and ethanol was added to provide optimal binding conditions. The lysate was then loaded onto the RNeasy silica gel membrane contained in the mini column. The RNA bound to the membrane and the contaminants passed through the mini column following centrifugation and were washed away with buffer. The mini column was then transferred to a new microcentrifuge tube and the RNA (~ 2 μg) was eluted in 30 μl of water and recovered after centrifugation.

After extraction, the mRNA concentration was measured by UV spectrophotometry (Jenway 6405 UV/visible spectrophotometer) at 260 nm (one optical density unit is equivalent to a concentration of 40 mg/ml of single stranded RNA) and was used immediately as a template for complementary deoxyribunucleic acid (cDNA) synthesis.

Primers design and sequences

Primers were designed to amplify the extracellular portion of the HLA-A*0201 molecule (bases 73 to 910). The trans-membrane region as well as the intracellular portion of the molecule were excluded because they tend to be hydrophobic and would have made the solubilisation and refolding of the molecule difficult; moreover, they are not needed for the molecule to bind the T cell receptor. Identical primers were used to amplify the extracellular portion of the HLA-A*0301 molecule, as there was only one base mismatch between the HLA-A*0201 and HLA-A*0301 sequences for each primer annealing site (Figure II.2).

The primers were designed to be complementary to bases of the HLA-A*0201 sequence to amplify in the Polymerase chain Reaction (PCR) and were modified to contain specific endonuclease enzyme cleavage sites (highlighted in Table II.3 and Figure II.2). The restriction sites were chosen to allow cloning in a protein expression

vector and encode for the start codon and that were necessary to define the DNA reading frame.

PRIMER SEQUENCES: HLA-A*0201 Polymerase Chain Reaction	
Forward oligo primer (5'-3'): 23 mers	Reverse oligo primer (5'-3'): 27 mers
GCACCATGGGCTCTCACTCCATG	CTGGGAAGACGGATCCCATCTCAGGGT
Nco I restriction site	Bam HI restriction site

Table II.3 Primer sequences: HLA-A*0201 Polymerase Chain Reaction (PCR)

Summary of oligo-primers length and sequence used to amplify the HLA-A*0201 and HLA-A*0301 molecules by PCR. Enzyme restriction sites were included in the primer sequence to allow cloning in a protein expression vector: Nco I (turquoise) and BamHI (yellow). The underlined codon encoded for methionine marking the start of transcription (see Figure II.2).

All DNA primers mentioned in this thesis were purchased from Amersham Pharmacia Biotech.

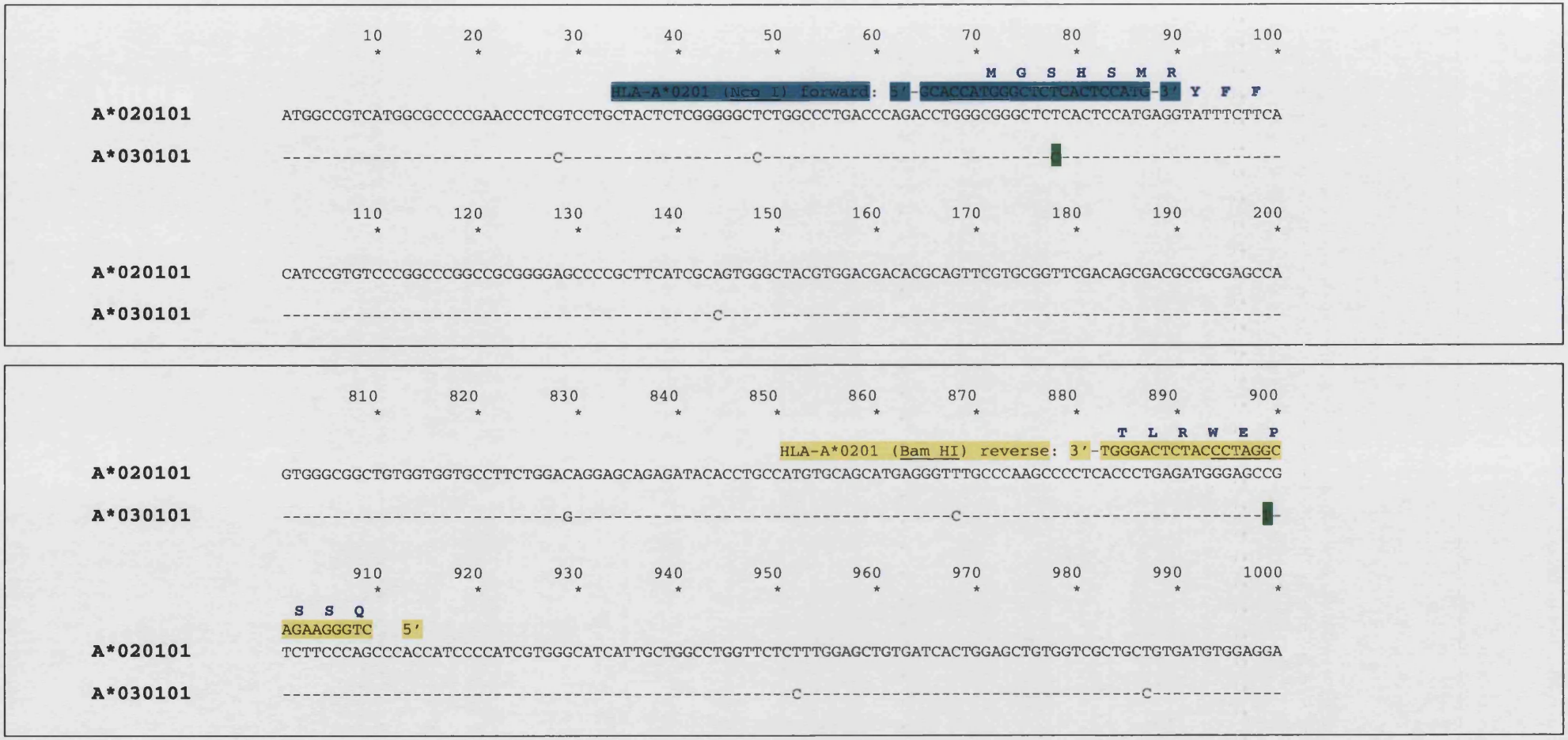

Figure II.2 HLA-A*0201 and HLA-A*0301 alignment and PCR primers

HLA-A*0301 was aligned with HLA-A*0201 as a reference using the alignment tool written by James Robinson for the IGMT/HLA database. The cloned sequence included residues 73 to 898 from the 1098 residues encoding the HLA-A*0201 molecule. The restriction enzyme sites included in the primers are underlined, the forward and reverse primers are highlighted in blue and yellow respectively, and the single mismatches between the two molecules in the primer sequences are highlighted in green.

Synthesis of complementary strand Deoxyribonucleic acid (cDNA)

cDNA was synthesised from template RNA using the Reverse Transcriptase Polymerase Chain Reaction enzyme (RT-PCR, with the Moloney Murine Leukaemia Virus Reverse Transcriptase or M-MLV-RT enzyme from Promega). This enzyme is an RNA-dependent DNA polymerase that was used to synthesise a first strand cDNA from a long messenger RNA template, in this case total cellular RNA. A volume of 10 µl (or ~ 1 µg) of RNA purified with the RNeasy kit from $5x10^6$ PBMCs was dispensed in a sterile RNase free microcentrifuge tube and 0.5 µg of random primers (Promega) per mg of mRNA sample were added in a maximum 15 µl final volume. The RNA was annealed to the random primers by heating the tube to 70°C for 5 min in a hot block to melt the secondary structure within the template. The tube was cooled immediately on ice to prevent the secondary structure from reforming and was then spun down briefly to collect all of the solution at the bottom of the tube.

The following components were then added in respective order:

M-MLV 5X Reaction Buffer	5 µl
dATP, 10 mM	1.25 µl
dCTP, 10 mM	1.25 µl
dGTP, 10 mM	1.25 µl
dTTP, 10 mM	1.25 µl
rRNasin® Ribonuclease inhibitor	25 units
M-MLV RT	200 units
Nuclease free water	25 µl

Table II.4 Components of the reverse transcriptase reaction

This table summarises all components of the RT-PCR reaction needed to obtain cDNA from purified cellular RNA.

The components of the reaction were mixed gently by flicking the tube. The tube was then incubated for 60 min at 37°C. The cDNA fragments obtained were used immediately as a template in the PCR reaction.

DNA gel electrophoresis

Agarose gels were used to separate DNA fragments of different size; they were prepared by mixing 0.8% electrophoresis grade agarose (Gibco BRL) with 50 ml of tris-acetic acid-EDTA (TAE: 0.04M Tris acetate, 1 mM EDTA) buffer (see buffer recipes) in a glass bottle (Duran) and melted in a microwave oven. The melted agarose was cooled down and poured into a sealed gel tray (Bio Rad). A comb was placed to form

wells, 5 µl of ethidium bromide (10 mg/ml) was added and the gel was left to solidify. A gel was placed in a mini SUB™ DNA cell (Bio Rad) that was then filled with TAE buffer. The combs were removed to load the DNA samples previously mixed with 2 µl of Orange G buffer (0.29% Orange G, 30% glycerol). A well was kept to load 5 µl of 1Kb Plus DNA Ladder™ diluted in Orange G (0.1 µg final concentration, Gibco BRL), then 200 volts were applied for about 40 min to induce DNA migration towards the positive electrode. DNA bands were then visualised in the gel under UV light and a digital photograph was taken.

Polymerase Chain Reaction (PCR)

Single stranded cDNA was obtained from a HLA-A*0201 and a HLA-A*0301 individual, it was however necessary to synthesise the second DNA strand to each cDNA sample before the PCR reaction could be performed as a double stranded DNA template was needed. This was included as a first cycle of amplification of the PCR reaction programme, using a low annealing temperature and identical primers to the main PCR reaction (for the primers sequences, see Table II.3).

The annealing temperature for both primers in the main PCR amplification was determined after the melting temperature (T_m) for each primer was calculated using the following formula:

$$T_m = 61.2 + 0.41\ (\%age\ GC) - 500/L$$

with %age GC being the percentage of guanosine (G) and cytidine (C) bases contained in the primer and L being the length of the primer.

The respective T_m calculated for each primer were (67°C) for the forward primer and (64°C) for the reverse primer. The lowest T_m was chosen and nine degrees were subtracted (as the primers were not an exact match to the sequence we wanted to amplify) to obtain the annealing temperature; therefore the final annealing temperature used in the reaction was 55°C.

The PCR reaction was performed using a higher quality DNA polymerase enzyme (Platinum Taq DNA polymerase, Invitrogen) as previous attempts with a regular DNA polymerase had resulted in coding errors that were detected by sequencing of the positive recombinant clones that had been selected. The following components were mixed in a sterile PCR tube:

Platinum DNA polymerase 10X buffer with 1.5 mM $MgCl_2$	10 µl
DNTP mix, 10 mM each	1 µl
Forward primer (6 pmol/µl)	5 µl
Reverse primer (6 pmol/µl)	5 µl
Platinum DNA polymerase	0.5 µl
cDNA template	1 µl
Nuclease free water to a final volume of	100 µl

Table II.5 Components of polymerase chain reaction

This table summarises all components of the PCR reaction needed to obtain amplified double stranded DNA from cDNA.

The reaction tubes, including HLA-A*0201, HLA-A*0301 and a negative control were then placed in a PCR machine (Primus, MWG-Biotech). The following programme was then applied:

Duration		Temperature (°C)	Effect
	5 min	95	Denaturation
5 cycles	1 min	94	Denaturation
	1 min	46	Low specificity annealing
	1 min	72	extension
30 cycles	1 min	94	Denaturation
	1 min	55	annealing
	1 min	72	extension
	10 min	72	Final extension

Table II.6 Polymerase chain reaction reaction

This table summarises all steps of the PCR reaction programme used to amplify double stranded HLA-A*0201 and HLA-A*0301 DNA.

A 20 µl sample of amplified DNA fragment was tested by gel electrophoresis to determine if the reaction had been successful and if the amplification was specific and produced a fragment of the expected size (Figure II.3).

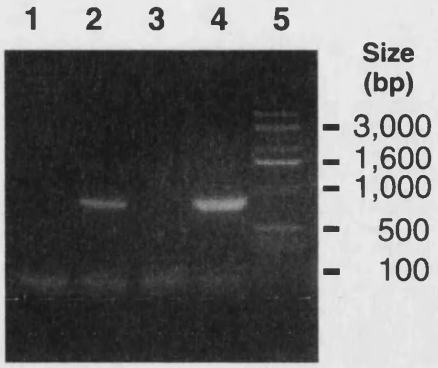

Figure II.3 HLA-A*0201 and HLA-A*0301 PCR amplification

20 µl samples of the amplified fragments were separated on 0.8% agarose gels. The following had been loaded: lane 1, no DNA template negative control; lane 2, product of HLA-A*0201 cDNA synthesis; lane 3, product of HLA-A*0201 cDNA synthesis, 1:100 dilution; lane 4, product of HLA-A*0301 cDNA synthesis. Lane 5 was reserved for the1 Kb DNA ladder. The bands were sharp and of expected size: around 840 base pairs (bp).

DNA mini-preparation and maxi-preparation

DNA mini-preparation

On average, 10 to 20 single colonies were tested to check whether they contained recombinant plasmids.

A single colony was inoculated in 5 ml of liquid culture medium containing the appropriate selective agent(s) and incubated overnight at 37°C in a loosely capped Universal container (Bibby Sterilin) placed in a shaking incubator. A 1.5 ml sample of culture was transferred to a microcentrifuge tube and spun for 1 min at 12,000 g at 4°C (the remainder of the culture was kept at 4°C to grow a larger preparation if one of the clones was recombinant). The medium was removed by aspiration and all following steps were performed on ice. The pellet was resuspended in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA) to lyse the bacteria and vortexed vigorously. Then 200 µl of freshly prepared Solution II (0.2 M NaOH, 1% (w/v) SDS, mixed immediately prior to use) was added and mixed gently by inversion. This was followed by the addition of 150 µl of Solution III (obtained by mixing 60 ml of 5M KoAc (potassium acetate), 11.5 ml of glacial acetic acid and 28.5 ml of water and obtain a 3M potassium-5M acetate final solution). After thorough mixing, the tube was centrifuged at 12,000 g for 2 min at 4°C. The supernatant was transferred to a fresh tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 6/6.8; BDH)

was added. After vortexing, the tube was centrifuged at 12,000 g for 2 min at 4°C and the supernatant was transferred to a fresh tube. The DNA was then precipitated by adding two volumes of ethanol to the supernatant at room temperature and vortexed. After 2 min of incubation at room temperature or alternatively 10 to 15 min at -20°C, the tube was centrifuged at 12,000 g for 5 min at 4°C. The supernatant was aspirated totally and the pellet was allowed to drain, before being rinsed with 70% ethanol. It was then allowed to air dry before resuspension in 50 µl double-distilled water.

DNA midi-preparation

The DNA maxi-preparation was essentially a scaled up version of the DNA mini-preparation. It was performed by growing a 50 ml bacterial culture overnight and the typical yield for this method was 3 to 5 µg of DNA per millilitre of original bacterial culture.

Measuring DNA concentration

DNA concentration was assessed by UV spectrophotometry at 260 nm (6405 UV/vis spectrophotometer, Jenway) which allowed a correlation of one optical density (OD) unit to 1 mg/ml of DNA.

DNA endonuclease restriction digest

Endonucleases are restriction enzymes that can be isolated from bacteria and cut DNA at a specific target site. The following enzymes (Table II.7), all at 10 U/ml (Gibco BRL) were used in this project to clone DNA fragments into plasmid vectors and to screen clones.

Endonuclease	Target sequence
Eco RI	5'-G↓AATT C-3' 3'-C TTAA↑G-5'
Bam HI	5'-G↓GATC C-3' 3'-C CTAG↑G-5'
Nco I	5'-C↓CATG G-3' 3'-G GTAC↑C-5'
Bgl II	5'-A↓GATCT-3' 3'-TCTAG↑A-5'

Table II.7 Endonucleases and restriction target sequences

This table summarises all endonucleases used to clone and screen positive clones encoding for the extracellular portion of HLA-A*0201 and HLA-A*0301 molecules and the bsp insertion in the pET 8 vector. Arrows indicate cleavage sites.

These enzymes were typically used by mixing 1 to 5 µl of DNA (constituting a maximum of 1 µg of DNA) with 2 µl of 10X React® 3 buffer (final concentrations: 50 mM Tris-HCl pH8.0, 10 mM $MgCl_2$, 100 mM NaCl; Gibco BRL) and 1 µl of restriction enzyme. The final volume was made up to 20 µl with double-distilled water. This was incubated for 1 hr at 37°C and the products of this digestion were separated by gel electrophoresis (Figure II.4).

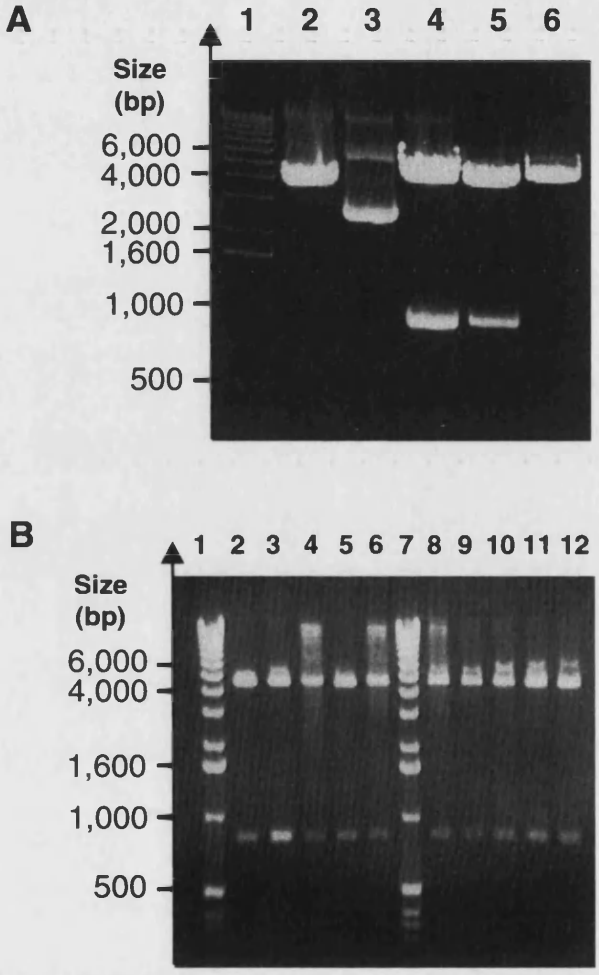

Figure II.4 Screening of HLA-A*0201 recombinant bacterial clones

10 µl samples of DNA mini-preparations were loaded and run on 0.8% agarose DNA gels after restriction digest with Eco RI (PCR 2.1 clones, panel A) or Nco I and Bam HI (pET 8 bsp clones, panel B). On panel A, two clones were positive for the 840 base pairs (bp) DNA fragment of interest; clone 4 was selected for subcloning into pET 8 bsp. On panel B, all the clones were positive for the Nco I/Bam HI 840 base pairs cloned fragment. A few positive clones were selected and tested for optimal protein expression.

DNA gel purification

Following endonuclease restriction digest, the DNA fragments that contained the coding sequence for the protein of interest (HLA-A*0201 or HLA-A*0301) were separated from the unwanted sequences (usually the vector backbone) by gel electrophoresis. The DNA was then purified from the agarose gel using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). A narrow gel slice containing the DNA band of the correct size, as seen under UV lighting, was cut with a sterile scalpel and transferred to a 1.5 ml microcentrifuge tube. The tube was weighed to determine the weight of the gel slice (the empty tube having been weighed previously and its known weight subtracted from the total). The slice was then cut into several smaller parts and 10 µl of capture buffer for each 10 mg of gel was added. The tube was closed and mixed by vortexing vigorously, then it was incubated at 60°C for 5 to 15 min until the agarose dissolved completely. The melted sample was transferred to a GFX column placed over a collection tube and incubated for 1 min to allow the DNA to bind to the glass fibre matrix. The sample was then spun through the column at maximum speed in a microcentrifuge and the eluate was discarded. 500 µl of wash buffer was applied to the column and the tube was centrifuged a second time. The column was then transferred to a fresh sterile 1.5 ml microcentrifuge tube, 50 µl of double-distilled water was applied directly to the matrix. The tube was incubated for 1 min at room temperature and centrifuged for 1 min at full speed to recover the purified DNA.

Plasmid Vectors and vector modification

Two different vectors, PCR 2.1 and pET 8, were used in our cloning strategy to ensure an efficient system and allowed a degree of versatility so that the constructs could be utilised in alternative experiments (PCR 2.1 and pET 8 maps can be found in the Appendix).

Initially, the products of PCR amplification were ligated into the PCR 2.1 vector (TA cloning kit, Promega). The product of PCR amplification of a DNA fragment by the enzyme DNA polymerase ends in an overhanging adenosine base at the end of each DNA strand. The PCR 2.1 vector has been designed and manufactured as a linear double stranded DNA fragment that ends with overhanging thymidine bases that are flanked by EcoR I restriction sites to allow for the screening of recombinant bacterial clones by single endonuclease digestion. The PCR 2.1 vector encodes for a short gene embedded in the poly-cloning site coding for the first segment of the β-Galactosidase

gene (Lac Z) that can complement the carboxy-terminal part of the enzyme encoded by E.coli to form an enzymatically functional protein. Addition of isopropylthio-β-D-galactoside (IPTG, Sigma) in the medium can trigger protein transcription by inducing the T7 promoter. If the Lac Z gene is not interrupted by a cloned DNA fragment, the bacteria will be able to process 5-bromo-4-chloro-3indolyl-β-D-galactoside (X-gal) and form colonies coloured by its blue degradation product. Consequently, the ligation of PCR fragments in PCR 2.1 was very efficient and after transformation in E.coli XL1B, allowed the production of large quantities of plasmid DNA that were tested by endonuclease digestion. This plasmid DNA was kept as stock and because the PCR 2.1 vector contained a cloning cassette with a range of restriction sites, this offered different sub-cloning options if needed for later projects.

The DNA fragment of interest was then subcloned into a modified protein expression vector pET 8 (Kind gift of Dr F.W Studier, now available commercially from Novagen under the name pET 3d). The DNA transcription and translation of genes ligated in pET vectors are placed under the control of strong bacteriophage T7 signals. The pET 8 plasmid vector had been modified by Chrissy Zamoyska in the laboratory. This involved a consensus biotinylation substrate peptide (bsp2) tag (chosen from a panel of candidate peptides described by Schatz in 1993) that was inserted at the end of the cloning cassette using DNA linkers (see Figure II.5). After ligation of the HLA coding fragment in this modified vector and verification by DNA sequencing, the encoded molecule was transcribed all the way through to the bsp tag and the transcription was ended by a stop codon. The biotinylation substrate peptide then became part of the translated protein.

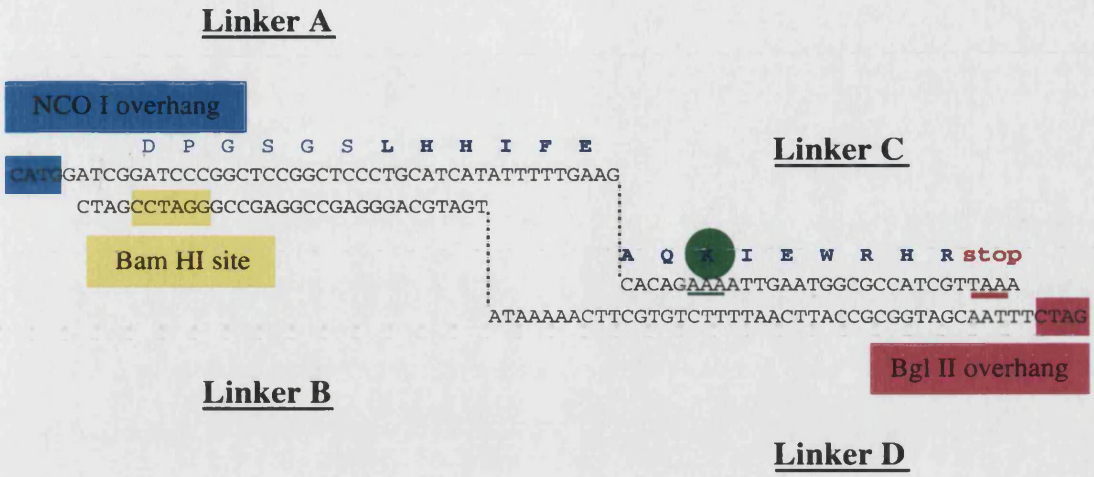

Figure II.5 Bsp tag: linkers sequences

The four complementary oligo-nucleotide linkers (A,B,C and D) were made to anneal and were then ligated into a Nco I/BamHI purified restriction fragment of the pET 8 vector (Bam HI and Bgl II overhang fragments are complementary, this also cancels this end Bam HI site from the pET 8 vector; see). Nco I and Bgl II overhangs are highlighted in blue and pink respectively. The bsp tag coded for the biotinylation substrate sequence (in bold blue letters) containing a lysine residue that was to be biotinylated (●) and was terminated by a stop codon (—).

The β-2 microglobulin molecule was cloned into the pHN1 ampicillin resistant vector, transformed into E.coli (strain XA90 f' LacIq1) and was kindly donated to our laboratory by Professor Don Wiley (Garboczi *et al*, 1992).

DNA Ligation, transformation into E.coli

DNA Ligation

Ligations of the original HLA-A*0201 and HLA-A*0301 PCR fragments into the PCR 2.1 vector were performed using the TA cloning® kit (Invitrogen). A volume of 1 to 5 µl of freshly amplified PCR fragment (~ 10 ng) was added to 2 µl of PCR® 2.1 vector (25 ng/µl), 1 µl of T4 DNA ligase (5 U/µl) and 2 µl of 10x Ligation Buffer in a microcentrifuge tube. The total volume was made up to 20 µl with sterile water and the tube was incubated overnight in a waterbath at 15°C. Some of the ligated DNA product was then used to transform competent bacteria and the remnants were kept frozen at -20°C.

HLA-A*0201 and HLA-A*0301 purified Nco I/Bam HI restriction fragments (5 to 10 µl) were ligated into the bsp2 modified and Nco I/Bam HI purified pET 8 vector

fragment with T4 DNA ligase and Ligation Buffer (both from Boehringer Mannheim) using an identical protocol.

Transformation of Escherichia coli (E. coli)

Competent E. coli bacterial strains XL1-Blue and BL21(DE3)pLys S were prepared following a classical calcium chloride membrane permeabilisation protocol and kept in aliquots at -80°C.

The XL1-Blue strain was transformed with the PCR 2.1 constructs to allow for the colour selection of recombinant colonies (see below). It was subsequently transformed with the bsp2 modified pET 8 constructs to produce sufficient amounts of plasmid DNA for the selection of recombinant colonies and the sequencing of the selected clones.

The BL21(DE3)pLys S strain was transformed with plasmid DNA from selected bsp modified pET 8 HLA-A*0201 or HLA-A*0301 constructs to express the extracellular portion of these proteins.

The competent cells were thawed slowly on ice, and a sterile 1.5 ml Eppendorf tube was cooled down on ice simultaneously. A volume of 200 µl of competent cells was gently mixed by inversion with 5 µl of ligated DNA mix. The competent cells and DNA were incubated on ice for 30 min, then heat shocked at 42°C for 3 min. 1 ml of bacterial liquid culture medium was added to the transformed competent cells and the tube was incubated at 37°C for an hour. Two different volumes (usually 50 and 200 µl) were then overlaid on Petri dishes containing selective culture medium with agarose.

Bacterial culture

Liquid bacterial culture medium

Liquid 2xYT culture medium for E.coli bacteria was prepared by solubilising 16 g of Peptone (Gibco BRL), 10 g of Yeast extract (Oxoid) and 5 g of Sodium Chloride (NaCl, BDH) in a litre of double distilled water (Select Bio water filter, Purite). The solution was sterilised by autoclaving before use.

Solid bacterial culture medium

Solid 2xYT bacterial culture medium was prepared in an identical fashion to the liquid culture medium, with the difference that 16 g of bacterial agar (Select Agar, Gibco BRL) per litre of medium was added before autoclaving. The solution was cooled down to 50°C, appropriate selection agents were added (if applicable) before it was poured

into sterile Petri dishes (Falcon Ten-twenty-nine™ Petri Dish, BD) and left to set before use or storage for up to two weeks at 4°C.

Bacterial plasmids and bacterial selection

Most recombinant plasmids encode for a gene that confers specific antibiotic resistance, so inclusion of the relevant antibiotic allows the recombinant colonies to be resistant, thus preventing other contaminating bacteria, which are antibiotic sensitive, from growing. The PCR 2.1 plasmid (Invitrogen) and the pET 8 plasmid both encode for an ampicillin resistance gene (Appendix VII and VIII). Thus, the bacteria following transformation with one of these recombinant plasmids were grown in medium containing a final concentration of 50 μg/ml of Ampicillin (Sigma).

To screen for recombinant PCR 2.1 colonies, 40μl of IPTG (20μg/ml) and 40 μl of X-gal (20μg/ml (Sigma) in dimethylformamide) were mixed, overlaid on bacterial agarose medium and left to dry before the product of the bacterial transformation was spread onto the agarose. After overnight incubation at 37°C, recombinant white colonies were selected to set up DNA mini-preparations and checked for recombination by endonuclease digestion (non-recombinant colonies were able to process X-gal and as a result appeared blue).

The BL21(DE3)pLysS E.coli strain was a constitutive host of the pLysS plasmid that provided an inhibitor of the T7 RNA polymerase enzyme. This prevented the transcription of the pET 8 plasmid encoded protein during the initial period of bacterial growth and also encoded for an ampicillin resistance gene. The BL21 strain also contained a chloramphenicol resistance gene. Therefore, the selection of positive recombinant bacterial clones in this strain was performed in medium containing both Ampicillin and Chloramphenicol (at a final concentration of 34 μg/ml, Merck). The BL21 host strain contains a chromosomal copy of the T7 RNA polymerase gene, and the DE3 lysogen encodes for the Lac repressor gene that represses the transcription of T7 RNA polymerase and the transcription of the target gene. Additionally, these bacteria contain the pLysS plasmid that encodes for the low-level expression of T7 lysosyme, which binds to T7 RNA polymerase and inhibits transcription. The two types of T7 RNA polymerase repression are counteracted by the addition of IPTG to the bacterial culture, which results in high transcription levels of the T7 RNA polymerase and target genes.

DNA sequencing

A few clones testing positive for the HLA-A*0201 or HLA-A*0301 DNA inserts after restriction digest and gel electrophoresis were sequenced to confirm that no mistakes had occurred during the PCR amplification and or cloning steps. Oligo nucleotide primers were designed to bind to the external section of the pET 8 vector cloning cassette (Amersham Pharmacia Biotech). The respective primer sequences are described in the following table:

PRIMER SEQUENCES: pET 8 sequencing	
Forward oligo primer (5'-3'): 20 mers	Reverse oligo primer (5'-3'): 20 mers
GGAGACCACAACGGTTTCCC	TGCTAGTTATTGCTCAGCGG

Table II.8 pET 8 primers for Sequencing

Forward and reverse primers used to sequence pET 8 plasmid DNA clones.

DNA purified by the DNA miniprep protocol (described previously) was sent to the HLA and Bio-informatics group at the ANRI, where the sequencing analysis for the selected clones was carried out. The sequencing reaction was performed in both directions by ABI-Prism big dye terminator chemistry and was run on an ABI 377 automated DNA sequencer. The resulting sequences were aligned with the Sequence Navigator Software and showed to be identical to the established HLA sequence for these antigens and was also in frame for translation in the pET 8 vector.

Synthetic peptides

Choice of peptides

Control peptides

Two control peptides were selected. The first control peptide was the influenza matrix (58-66) HLA-A*0201 specific peptide (amino-acid sequence: GILGFVFTL), shown to bind HLA-A*0201 with high affinity (Morrison *et al*, 1992). This specific peptide was used as a positive reference for the refolding and synthesis of the HLA-peptide complexes. The second control peptide, selected for its HLA-A*0201 binding properties was the minor antigen HA-1 HLA-A*0201 specific peptide (amino-acid sequence: VLHDDLLEA; Ren *et al*, 2000). Specific T cell responses restricted to this peptide are only observed in HA-1 mismatched patients (Mutis *et al*,1999) and therefore would not have induced a T cell response in healthy individuals. It was used as a negative HLA/peptide tetramer control staining.

CMV study

Three peptides from the immunodominant pp65 protein were selected for this study. They were synthesised by fluorenylmethoxycarbonyl (F-moc) chemistry and purified by high performance liquid chromatography (HPLC) before being dessicated and stored at -20°C. They were all shown to bind the HLA-A*0201 molecule and could therefore be potentially presented to T cells. The peptide sequences are summarised in the following Table:

Peptide	Amino acid sequence	pp 65 fragment (amino acids)
AE 42	NLVPMVATV	495-503
AE 44	VLGPISGHV	14-22
AE 45	MLNIPSINV	120-128

Table II.9 Cytomegalovirus pp 65 specific peptide sequences

This table summarises the three peptides that were selected to be refolded with HLA-A*0201 and be made into HLA/peptide tetrameric complexes. The leucine (L) and valine (V) anchoring residues are highlighted at positions 2 and 9 of the HLA-A*0201 molecule peptide binding groove.

CML study

A single peptide from the BCR/ABL b3a2 junction peptide was predicted as a potential binder to the HLA-A*0301 molecule. The amino-acid sequence of the predicted peptide was KQSSKALQR. It was solubilised in RPMI 1640 at 1mg/ml and stored at -20°C.

Solubilisation of peptides

Dessicated peptide stocks were kept at -20°C, and were solubilised in a 50% dimethylsulfoxide (DMSO) solution. A quantity sufficient for each HLA refolding was dissolved immediately before use in a total volume of 1 ml of 50% DMSO and peptide used in cell stimulation experiments was dissolved in 50% DMSO at 1 mg/ml, and stored at +4°C.

Biochemistry

Protein expression and maxi-preparation

A single colony, or a scraping from a frozen glycerol stock, was inoculated into 10 ml of liquid bacterial culture medium with the relevant antibiotic selection and incubated overnight in a shaking incubator at 37°C.

The overnight culture was then inoculated into one litre of autoclaved bacterial culture medium containing the appropriate selection and was placed in a shaking incubator at 37°C until the culture was in exponential growth and had reached an optical density of 0.4 as measured by spectrophotometry at 550 nm. Protein expression was then induced by adding IPTG to the culture at a final concentration of 1 mg/ml. After 4 hrs of further incubation, the bacteria were harvested and transferred to a one litre centrifuge bottle and centrifuged at maximum speed for 30 minutes at +4°C (Beckman JB-6). The supernatant was disinfected and discarded and the pellet was resuspended in 10 ml of Bacterial Lysis buffer (50 mM Tris-Cl pH 8.0, 1 mM EDTA, 25% (w/v) sucrose, filter sterilised) and transferred to a fresh 50 ml Falcon tube (BD). From this point, all steps were performed on ice. The tube was centrifuged at 4000 g for 10 min at 4°C to remove any remaining medium and the supernatant was discarded. The pellet was resuspended with 10 ml of B-PER® Bacterial Protein Extraction Reagent (Pierce) and 2.5 ml of lysosyme (10 mg/ml freshly prepared stock, Sigma) was added. The tube was then frozen, thawed to fully lyse the bacteria and sonicated (five 15 seconds bursts of sonication at 45 seconds intervals) on ice. The sample was spun at 10,000 g at 4°C for 10 min. The insoluble proteins recovered as inclusion bodies were washed once in 10 ml of detergent buffer (0.2 M NaCl, 1 % (w/v) deoxycholic acid, 1 % (v/v) Nodinet P 40, 20 mM Tris-Cl pH 7.5, 2 mM EDTA pH 8.0, filter sterilised) with phenylmethylsulphoxyde (PMSF) at a final concentration of 1 mM. They were then washed three to four times with 10 ml of Triton wash buffer (0.5 % (v/v) Triton X 100, 50 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0) until a milky white pellet was obtained. After the final wash the supernatant was discarded and the pellet resuspended in 5 ml of the following buffer: 50 mM Tris and 1 mM EDTA, 1 mM of PMSF and 2 mM dithiothreitol (DTT). The inclusion bodies were then stored at -20°C or solubilised in deionised 8 M urea buffer (8 M deionised urea with 10mM Tris and 150 mM NaCl) before use in a refolding solution. The 8 M urea solution was deionised

by incubating 5 g of Amberlite ion exchanger (Merck) overnight in 200 ml of solution, then the beads were removed by filtration.

Protein gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The size of the expressed proteins was checked by SDS-PAGE. The theoretical molecular weight of the expressed proteins was determined by submitting the full amino acid sequence to the Biopolymer Calculator Results v4.1.1 software, which can be accessed freely at the following web address: http://paris.chem.yale.edu/cgi-bin/extinct.pl. For the cloned HLA-A*0201 or HLA-A*0301 extracellular portion with bsp, the molecular weight was estimated at around 35,000 Dalton (Da). The full sequence of the human β-2 microglobulin molecule was cloned (gift from Don Wiley) and its molecular weight was estimated to be 12,000 Da, as reported by Garboczi *et al*, 1992.

Fifteen percent acrylamide gels were prepared in a Mighty Small multi-gel caster (Hoefer scientific Instruments) and were composed of a separating gel matrix that allowed separation of the proteins according to their size; butan-2-ol was laid over the separating gel until it set to create an even surface). When the gel was set, a stacking gel layer was poured above it and loading wells were created by insertion of a gel comb. Both gels were made by mixing the relevant upper gel buffer (0.4 % (w/v) SDS, 0.5 M Tris-Cl, adjust the pH at 6.8, filter sterilised and kept at 4°C) or lower gel buffer (0.4% (w/v) SDS, 1.5 M Tris-Cl, pH adjusted to 8.8, filter sterilised and kept at 4°C) with the following components (sufficient for four gels):

	Separating gel	Stacking gel
Upper gel buffer	-	2.5 ml
Lower gel buffer	7.5 ml	-
30% acrylamide-0.8% Bisacrylamide (Protogel, National Diagnostics)	15 ml	1.5 ml
double distilled water	7.5 ml	6 ml

Table II.10 Composition of SDS-PAGE gels

This table summarises the volumes of the different components mixed to cast four 15% acrylamide SDS-PAGE gels in a Mighty Small multi-gel caster (Hoefer Scientific Instruments). The following solutions were added to polymerise both gel layers: 10 µl of N-N-N'-N' tetramethylethylene diamine (TEMED, BDH) and 100 µl of10% ammonium persulphate (APS, BDH).

The samples to be tested were transferred to 1.5 ml microcentrifuge tubes and mixed with 5 µl of SDS sample buffer (2% (w/v) SDS, 0.0625 M Tris-Cl, 10% (v/v) Glycerol, 5% 2-mercaptoethanol, 0.01% (w/v) bromophenol blue). The lids were pierced and the samples were denatured by incubation at 100°C for 5 min. The samples were then loaded into the wells of the pre-cast gels with one lane being used to load 5 µl of BenchMark™ pre-stained protein ladder (9,300 to 172,600 apparent molecular weight range, Gibco BRL). The gels were run in a Mighty Small II gel tank (Hoefer Scientific Instruments) in SDS-PAGE buffer (0.1 % (w/v) SDS, 0.025 M Tris-Cl, 0.192 M Glycine, filter sterilised) for 30 to 40 min at 30 mA. They were consequently stained with Coomassie brilliant blue, vacuum dried on Whatman paper and photographed or scanned (Figure II.6).

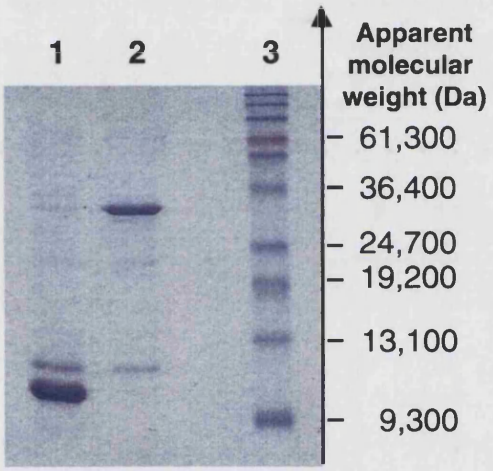

Figure II.6 SDS-PAGE analysis of large scale protein expression

Inclusion body preparation aliquots (20 µl) of human β-2 microglobulin (lane 1) and HLA-A*0201(lane 2) were dissolved in 8 M urea, and after the aggregated elements were discarded, were run on a 15% acrylamide SDS-PAGE gel. The apparent molecular weight of the molecules was compared to a pre-stained marker in lane 3 (size indicated in Dalton).

Moreover, this assay allowed for a qualitative assessment of the purity of the protein preparation by checking for contaminating bands on the SDS-PAGE gel. This was necessary to ensure that the contaminating proteins (usually encoded in the E.coli genome or in the plasmid vector) were present in small enough amount so that they would not interfere in the refolding reaction.

Native protein gel electrophoresis

This was performed after the synthesis of HLA-peptide complexes and their biotinylation (see below). Identical gel casting units and tanks were used for the native gels as for the SDS-PAGE gels. Native gels were composed of separating and stacking layers that was prepared using the following recipe:

	Separating gel	Stacking gel
1.5 M Tris-HCl, pH 8.8	7.5 ml	-
1 M Tris-HCl, pH 6.8	-	1.25 ml
30% acrylamide-0.8% Bisacrylamide (Protogel, National Diagnostics)	8 ml	1.7 ml
double distilled water	14.5 ml	6.8 ml

Table II.11 SDS-PAGE gel recipe

This table summarises the volumes of the different components mixed to cast two 8% acrylamide native gels in a Mighty Small gel caster (Hoefer Scientific Instruments).

As mentioned previously, 100 µl of APS and 10 µl of TEMED were added to induce gel polymerisation. The gels were pre-run in native gel electrophoresis buffer (24.8 mM Tris, 192 mM Glycine) at 150 volts for 30 min to remove any excess salt that could influence the separation. The samples were mixed with 5 µl of native gel sample buffer (50 mM Tris-Cl pH 6.8, 10% Glycerol, 0.01% (w/v) bromophenol blue) and were run in native electrophoresis gel buffer at 200 Volts until the bromophenol blue dye reached the end of the gel. The gels were then stained with Coomassie brilliant blue.

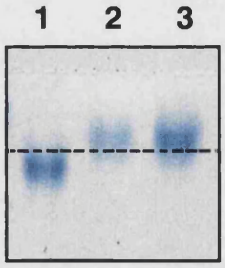

Figure II.7 Native gel shift assay

HLA-A*0201/CMV/biotin was incubated at a 4:1 molar ratio with streptavidin for an hour on ice. A native gel shift analysis was performed to confirm the level of biotinylation of the complexes: lane 1, HLA-A*0201/CMV/biotin alone; lane 2, streptavidin alone; lane 3, HLA-A*0201/CMV/biotin complexed with streptavidin. In lane 3, an 80 to 90% shift of the refolded HLA band to the level of the strepatvidin band is observed.

By mixing a sample of refolded and biotinylated HLA-peptide complex with the respective 4:1 molar ratio of streptavidin, the level of biotinylation of the complexes could be assessed and therefore taken into account when calculating how much fluorescently labelled streptavidin to be added.

Coomassie brilliant blue staining

SDS-PAGE and native protein gels were stained for 20 mins with gentle shaking in Coomassie blue staining solution (50% methanol (v/v), 10% glacial acetic acid (v/v), 0.25% (w/v) Coomassie brilliant blue G 250 in water). The gels were then destained in Coomassie destain solution (16.5% (v/v) methanol, 5% glacial acetic acid in water) that was changed frequently over 4 to 8 hrs. Destained gels were then photographed and dried on 3 mm chromatography paper (Whatman) using a Bio Rad gel drier (model 583).

HLA class I refolding

The refolding of the extracellular portion of HLA-A*0201 and HLA-A*0301 with peptide and β-2-microglubulin was performed using a dilution protocol. A dilution refolding buffer was prepared by combining the following in a final volume of 200 ml: 100mM Tris-HCl pH8.0, 400 mM L-arginine-HCl, 2mM EDTA, 5 mM reduced Glutathione, 0.5 mM oxydised Glutathione and 0.5 mM PMSF. The pH was adjusted to 8.0 and the buffer was cooled down to 4°C before adding the solubilised HLA and β-2-microglobulin chains and peptide at the following concentrations:

Solubilised protein	Final concentration
HLA-A*0201 or HLA-A*0301	1 mM
β-2-microglobulin	2 μM
Peptide	10 μM

Table II.12 Components of dilution refolding

This table summarises the concentration of the different components mixed in the dilution refolding for HLA class I molecules.

The solubilised HLA-class I heavy chain, β-2 microglobulin and peptide were added separately and drop-wise to the buffer while stirring simultaneously. The refolding mix was incubated with stirring at 4°C for 48 hrs. The product of the refolding was then transferred to 50 ml sterile Falcon tubes and centrifuged at 2,000 g for 15 min at 4°C to pellet any aggregated material. The supernatants were then transferred to an ultrafiltration stirred cell (8400 model, Amicon) and concentrated down to 20 ml through a microfiltration membrane of 10,000 molecular weight cut off (YM 10, 10,000

MWCO, Millipore) by applying gas pressure. The product of the refolding mix was then transferred to a Vivaspin 20 ml concentrator (10,000 MWCO, Vivascience) and further concentrated down to 1 ml or less by centrifugation (2,000 g at 4°C).

The refolded and concentrated product was kept at 4°C and purified by fast protein liquid chromatography (FPLC) gel filtration within 12 to 24 hrs.

Biotinylation reaction

Biotinylation of the biotin substrate peptide target sequence expressed at the C–terminal portion of the HLA class I molecules was performed using in-house bacterially expressed Bir A enzyme. This enzyme catalyses the formation of biotinyl-5'-adenylate from adenosine tri-phosphate (ATP) and biotin, and in E.coli, it transfers the biotin from the biotinyl-5'-adenylate to a particular lysine residue in only one protein. This allowed targeted biotinylation of the HLA molecules. The biotinylation carried out with the in-house Bir A was comparable in efficiency to commercially available enzyme (Avidity). The pure fraction containing correctly refolded HLA (in a 20 mM Tris, 5 mM NaCl, pH 8.0 buffer) was concentrated down to 200 µl. The reaction mix consisted of 8 parts of refolded HLA solution, one part of Biomix A buffer (0.5 M bicine, pH 8.3), one part of Biomix B buffer (100 mM ATP, 100 mM MgOAc, 400 mM biotin) as well as 10 µl of Bir A enzyme (1mg/ml). An appropriate amount of Complete™ protease inhibitor cocktail (Boehringer Mannheim) was added to the mix. The reaction was incubated overnight at RT, and the refolded and biotinylated product was purified from the excess biotin by FPLC gel filtration immediately thereafter.

FPLC gel filtration purification

Purification of refolded HLA molecules and refolded/biotinylated molecules was performed by FPLC gel filtration on an Äkta purifier instrument using a XK 16/70 column packed with 100 ml of Superdex™ 75 matrix (composed of dextran covalently linked to highly cross-linked agarose, Pharmacia). This system allowed the separation of proteins depending on their molecular weight. The column was equilibrated with buffer before the sample was loaded, a maximum pressure of 0.5 MPa was set on the instrument, and the pump was set to run at 1 ml/min. All samples were filtered by centrifugation through a 0.2 µm mini-filter (Vivaspin 0.5 ml, Vivascience) before loading in the injection loop. The elution of the proteins was programmed using the IBM operating system 2 (OS 2) to run the Unicorn 3.10 software and was carried out over one and a half column volumes. At the end of the column, the UV absorbance was

measured to determine where the proteins were eluted and 3 ml fractions were collected (Figure II.8). Prior to biotinylation, the refolded product was purified in a buffer containing a low level of salt (20 mM Tris, 5 mM NaCl, pH 8.0) in order to combine a buffer exchange to the purification procedure. This was necessary as the Bir A enzyme is inhibited by high salt concentrations. However the refolded product after biotinylation was purified in a buffer with physiological levels of salt (20 mM Tris, 150 mM NaCl, pH 8.0) as it would become the final storage buffer for refolded/biotinylated HLA molecules.

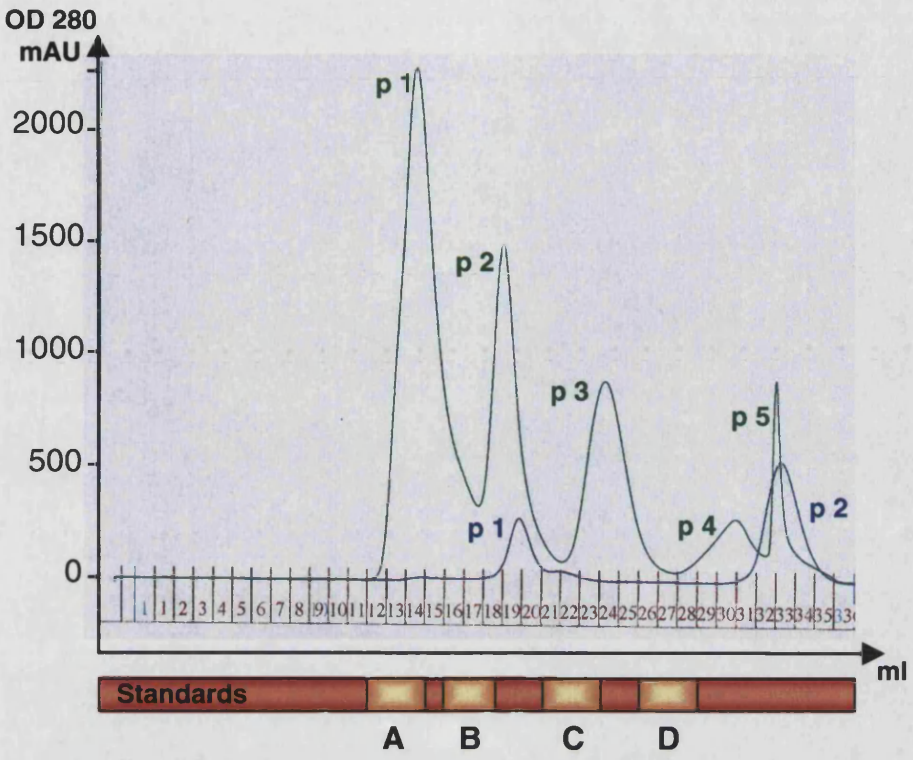

Figure II.8 FPLC purification traces: after refolding and after biotinylation

FPLC purification traces: after refolding of HLA-A*0201 with β-2 microglobulin and CMV peptide AE42, — curve; after biotinylation of a third of the refolded product from the second green peak (labelled p2, — curve. Fractions of 3 ml were collected (—) and the relative absorbance (milli-absorbance units, mAU) was measured at 280 nm to detect proteins throughout the run as they were leaving the separation matrix (— and — curves). The peak resolution was compared to the resolution of standard proteins separated with the same protocole (▬ standard markers): A, blue dextran, $2x10^6$ Da; B, Albumin, 66,000 Da; C, carbonic anhydrase, 29,000 Da; D, cytochrome C,12,400 Da. This allowed the determination of the peaks composition: purification after refolding —: p1, aggregated material; p2, refolded HLA; p3, β-2 microglobulin alone; p4, Arginine (present in refolding mix and absorbing at 280 nm); p5, remaining peptide; purification after biotinylation —: p1, refolded and biotinylated HLA molecule; p2, remaining biotin.

Measurement of protein concentration

Protein concentration in solution was measured using the Micro BCA protein assay reagent kit (Pierce) that uses bicinchoninic acid (BCA) as the detection reagent for reduced copper ions formed by the reaction of proteins with copper. One cuprous ion (Cu^{+1}) chelates two molecules of BCA and produces a purple water soluble complex characterised by a strong absorbance at 562 nm that is linear with increasing protein concentrations.

Briefly, serially diluted standards of bovine serum albumin (BSA) were prepared as well as diluted samples from the solutions to be tested. 500 µl of each standard dilution or unknown sample were dispensed in labelled tubes (5 ml round bottom tubes, Thermo Life Science), a 500 µl blank consisting of double distilled water was also prepared. Then 500 µl of mixed working reagent (containing the copper and BCA solutions, Pierce) was added to each test tube. After incubating for 1 hour at 37°C, the tubes were left to cool at room temperature and the absorbance was measured by transferring the contents of each tube to a plastic cuvette (1 ml plastic cuvettes, Jencons PLS) and reading the absorbance at 562 nm. The reading from the blank tube was subtracted from all other readings and the BSA standard curve was plotted against its concentration using the KaleidaGraph™ 3.0 Software. Finally, the protein concentration of the samples (refolded and biotinylated HLA molecule for example) was determined using the standard curve to establish the corresponding concentration.

HLA-Tetramer complexes

Avidin/Biotin interaction, tetramerisation

The biotin molecule has the ability to form a strong non-covalent interaction with the avidin molecule has four binding sub-units thus forming a tetramer. When a given molecule is tagged with a biotin molecule, the former can then be tetramerised by incubation with avidin or streptavidin at a molecular ratio of four to one respectively.
The extracellular portions of the HLA-A*0201 and HLA-A*0301 molecules were produced and refolded with specific nine mer peptides and β-2-microglobulin as described previously. These monomeric molecules were then biotinylated and tested by Dot-Blot and/or ELISA as described below to confirm the correct refolding and biotinylation of the complexes. They were then tetramerised by adding labelled streptavidin and formed a reagent that could specifically bind its T cell receptor ligand. Streptavidin (avidin purified from the microorganism Streptomyces avidinii) conjugated to the fluorescent markers phycoerythrin (PE) or alternatively allophycocyanin (APC) was purchased from Biogenesis and used to tetramerise HLA/peptide molecules (See Figure II.9, next page). Tetramerisation of these molecules was necessary to increase the avidity of the complexes for the T cell receptor and establish a relatively long lasting and stable interaction. This allowed the visualisation of these complexes and the detection of T cells specific for a particular HLA/peptide combination by fluorescence microscopy or flow cytometry.

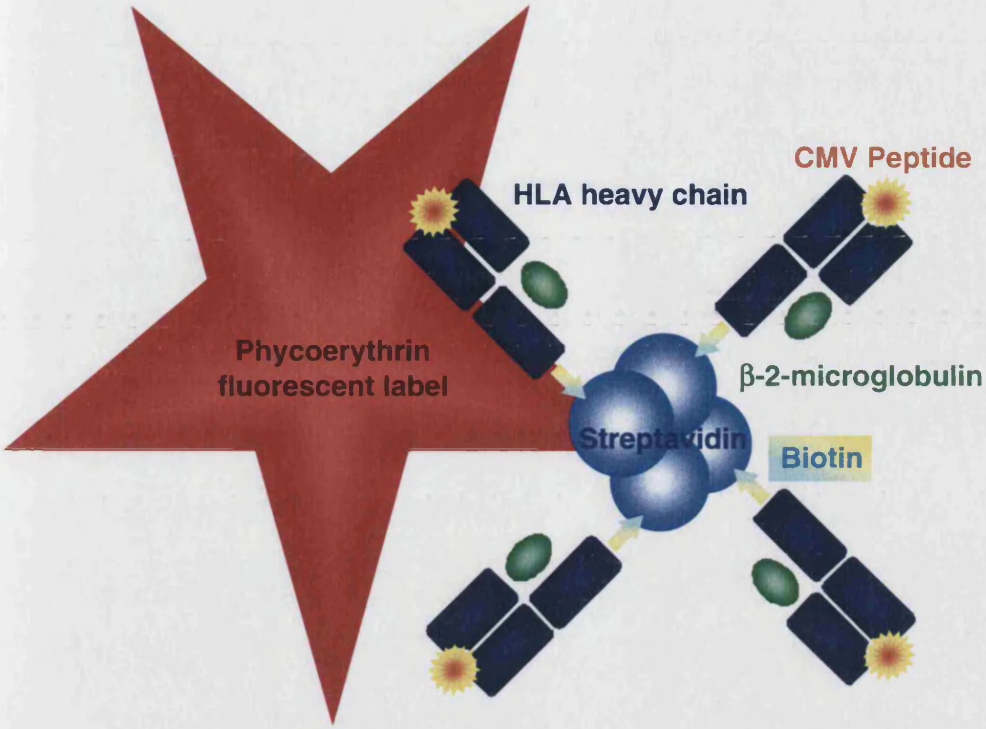

Figure II.9 HLA/peptide tetramer molecule composition

HLA/peptide tetramer molecules are composed of four biotinylated HLA molecules refolded with a peptide and β-2-microglobulin. These can bind streptavidin conjugated to a fluorescent label at 4:1 molar ratio.

HLA class I monomer and tetramer reagent storage

HLA class I monomeric and tetrameric complexes (in a10 mM Tris, 150 mM NaCl, pH 8.0 buffer) were mixed with 10% (v/v) of sterile glycerol. Aliquots were stored at -20°C.

HLA/peptide monomers were defrosted and tetramerised on ice by adding the conjugated streptavidin in small volumes over a couple of hours to favour the formation of tetramers. The tetramers were then aliquoted to be frozen and stored or they were used as a 4°C working stock.

Immuno-assays

Immuno-assays are all based on using specific immunoglobulins (or antibodies) and immunoglobulin combinations to detect a specific protein (or antigen) present in a biological sample. Immunoglobulins can be raised against a specific human protein by immunising an animal (often a mouse) with the molecule that is to be targeted. The purified antibodies can then be either conjugated to a labelled molecular marker or immobilised on a solid surface (nitro-cellulose membrane or immuno-plate) and will allow the detection and quantification of their specific antigen on the cell surface.

Characterisation of cell surface markers with fluorescent antibodies

The surface of cells is characterised by the presence of a high numbers of trans-membrane molecules that can bind to receptors on the surface of neighbouring cells or tissues.

The different fluorescent conjugates and antibodies to various lymphocyte cell surface molecules are summarised in the following tables:

name	Full name	emission colour, peak (nm)
FITC	fluorescein isothiocyanate	green, 520
PE	phycoerythrin	yellow, 576
PerCP	peridinin chlorophyl protein	red, 677
PerCP-Cy5.5	PerCP- cyanin 5.5	long red, 695
APC	allophycocyanin	red, 660

Table II.13 Antibody fluorescent conjugates

This table summarises the emission peak of different fluorescent components conjugated to antibodies (emission peaks obtained after excitation by an argon ion laser emitting light at 488 nm (or a red ion laser for APC)).

Directly conjugated antibodies were purchased from BD-Pharmingen and were used to stain cells at the recommended concentration for 30 min at 4°C in staining medium. Up to four different colours were detected simultaneously at the surface of a single cell. The unbound antibodies were washed off by addition of RPMI and centrifugation at 1,000 g. Samples were then re-suspended in fixative prior to analysis by flow cytometry. The specificity of the different antibodies used in this study are described in the following table:

Name	Protein target	Positive population phenotype
CD3	T cell receptor complex	T cells
CD8	T cell co-receptor	cytotoxic T cells
CD25	Interleukin 2 receptor	Activated T cells
CD27	T cell surface receptor	Activated T cells subset
CD95	Fas "death domain"	Activated T cells
CD45 RO	Leucocyte common antigen, RO isoform	Memory T cells subset
CD69	T cell surface molecule	Recently activated T cells
CCR7	CC chemokine receptor 7	T cells

Table II.14 Antibodies to lymphocyte surface molecules

This table summarises the different mouse-anti-human cell surface marker antibodies used to characterise different subset of T cells by flow cytometry.

Cross-linking and delivery of a specific co-signal

Biotinylated antibodies were purchased from BD-Pharmingen (mouse anti-human CD3, CD28 and CD95) or R&D systems (goat anti-human CD40L and 4-1BB) and were used to make HLA/peptide-antibody chimera tetramers or chimeramers. This consisted of first synthesising biotinylated monomeric HLA/peptide molecules as described in the previous section then in mixing these at 4°C with a co-stimulatory biotinylated antibody of choice at a respective three to one molecular ratio. The mix was then tetramerised as described in the HLA/peptide tetramer complexes paragraph.

The specificity of the different biotinylated antibodies used in these assays are described in the following table:

Name	Protein target
CD3	T cell receptor complex
CD28	T cell co-receptor, activation
CD40L (CD154)	T cell co-receptor, activation
4-1BB (CD137)	T cell surface receptor, survival
CD95	Fas "death domain"

Table II.15 Biotinylated antibodies to T lymphocyte surface molecules

This table summarises the different biotinylated anti-human cell surface marker antibodies (mouse or goat) used to synthesise HLA-peptide/antibody chimera tetramers or chimeramers.

These chimeramer complexes were used as an antigen specific stimulus and co-stimulus in cell culture assays at a concentration of 1µg/ml of culture medium for $2x10^6$ PBMCs.

Assessment of cellular apoptosis

The assessment of cellular apoptosis in cellular activation or cytotoxicity assays was performed with the intracellular caspase detection kit Apostat (R&D systems).

A group of caspase enzymes are part of a signalling cascade in the cell that can be triggered through different pathways, and once activated mediates programmed cell death or apoptosis. The substrate preferences and specificities of caspases have been exploited for the development of cell permeable peptides (conjugated to a fluorescent molecule, here FITC) that compete for caspase binding and therefore allow for the detection of active caspases by flow cytometry.

Cells were stained directly during the last 30 min of culture at 37°C prior to the assessment of apoptosis by adding 10 µl of Apostat reagent per 1 ml of culture medium. Any unbound reagent diffused out of the cells. The cells were then transferred to a sterile tube and washed with 4 ml of PBS to remove any unbound reagent. The cells were then resuspended in PBS and analysed by flow cytometry.

Protein Dot-Blot

After protein refolding and purification, several peaks were eluted from the Superdex column and were tested by immuno-labelling to confirm which fraction contained the correctly refolded HLA molecule. This was done using either by protein dot-blot or ELISA analysis.

Triplicate 50 µl samples were taken from each the FPLC elution peaks and were deposited onto a pre-wet labelled (with distilled water, then with PBS) Hybond™ ECL nitrocellulose membrane (Amersham Life Science) using a vacuum dot-blotter (Hybri-dot Manifold, Bethesda Research Laboratories). Each protein dot was washed twice with 200 µl of PBS before the membrane was transferred to a 50 ml Falcon tube (BD) filled with blocking buffer (PBS, 0.05% (v/v) Tween 20, 1% (w/v) BSA). This was incubated on a rolling cylinder overnight at 4°C. The membrane was then washed three times for 15 mins in PBS-T buffer (PBS with 0.05% (v/v) Tween 20). The triplicate samples were then cut into three identical membrane strips to be incubated with 5 µg/ml of different primary antibodies (either W6/32 or BB7.2 were used for HLA-A0201 while only W6/32 could be used for HLA-A0301) in 15 ml of PBS-T. The tubes were incubated for an hour at RT on a rolling cylinder. The antibodies used to test the blots and their role are described in the following Table:

Name	Specificity
Normal mouse serum	Negative control
W6/32	Conformational HLA class I
BB7.2	HLA-A2
Gap-A3	HLA-A3
BBM1 or ME-1	β-2 microglobulin

Table II.16 Dot blot: control and test antibodies

This table summarises the control and specific antibodies used to test the FPLC eluted fractions for refolding of the HLA molecules.

The samples were washed three times in PBS-T for 15 min at RT and they were incubated for an hour with a second layer goat-anti mouse antibody conjugated to the peroxidase enzyme (1:2,500 dilution, Sigma) in PBS-T. The strips were then washed three times for 15 min with PBS-T at RT, and the presence of the peroxidase on the blot was revealed by incubating the membrane in ECL™ chemi-luminescent reagent for 45 sec (ECL™ reagent kit, Amersham Pharmacia Biotech). The nitrocellulose membrane was then wrapped and exposed to X-ray photographic film (MXB film, GRI autoradiography blue sensitive, Kodak). A typical dot-blot film is represented on Figure II.10.

Biotinylation was checked in a similar way, with just a one step antibody incubation of the blot with Streptavidin coupled to the peroxidase enzyme.

Although good results were obtained with the dot-blot technique, it was quite time consuming, occasionally failed and required to be repeated. As any delay before storage or tetramerisation of the monomers might impair the quality of the resulting tetramer reagent, we decided to modify the dot-blot test for an ELISA approach, which would be a faster and more reliable method to test for refolding and biotinylation.

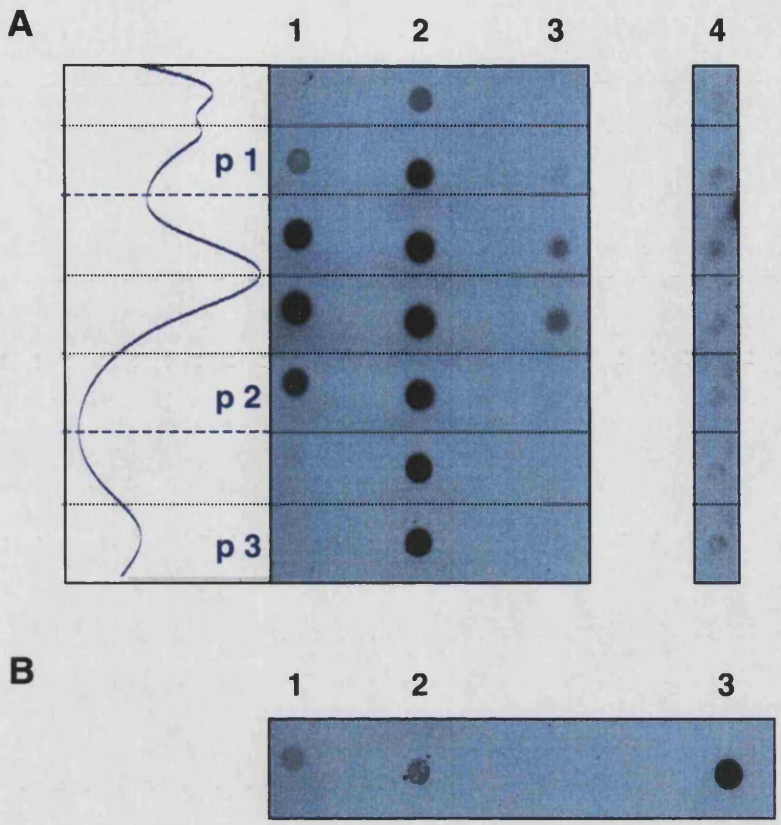

Figure II.10 Dot Blot analysis of peaks post refolding and post biotinylation

After refolding of the HLA-A*0201 molecule with β-2 microglobulin and the AE44 peptide, FPLC purification peaks (panel A, p1, p2 and p3, —) were blotted on a nitrocellulose membrane (see Chapter II). After blocking the membrane, the samples were probed with (panel A): W6/32 HLA class I conformation specific antibody, lane 1; BBM 1 β-2 microglobulin specific antibody, lane 2; BB7.2 HLA-A2 specific antibody, lane 3; normal mouse serum control, lane 4. Samples from p2 are positive for all antibodies and negative for the control confirming correct refolding of the HLA molecule. After biotinylation (panel B), a sample from the refolded and biotinylated peak was tested against a sample from p2 prior to biotinylation. Both samples and a negative control were probed with streptavidin-peroxydase: 1, normal mouse serum negative control; 2, p2 prior to biotinylation; 3, refolded and biotinylated peak. It can be noted that a small level of biotinylation is detectable in the second peak sample prior to the biotinyation reaction. This is likely to be due to biotinylation occuring at the time of bacterial expression, as the Bir A enzyme is constitutive of E.coli bacteria.

ELISA

The ELISA method was developed by one of the laboratory personnel as a more reliable and faster way to test FPLC eluted fractions for correct refolding and biotinylation of HLA-peptide monomers.

Briefly, on the day prior to the purification of a refolded and biotinylated HLA preparation, a Maxisorb immuno-plate (Nunc) was coated with W6/32 antibody at 5 μg/ml (100 μl per well). After incubating the plate for two hours at 37°C, the antibody solution was discarded and replaced by 100 μl of blocking buffer. This was incubated overnight at 4°C, then the blocking buffer was discarded and the plate was rinsed four to five times with PBS. Samples were then added (5 to 20 μl in a final volume of 100 μl of PBS) and serial dilutions performed, keeping a blank well (negative control) at he beginning of each row of samples. The plate was incubated at RT for an hour and washed five times with PBS. A second antibody layer consisting of 100 μl of rabbit anti-human β-2 microglobulin (1:5,000 in PBS, Dako) was then applied. It was incubated at RT for 20 min and then the unbound antibodies were washed off. A goat anti-rabbit antibody conjugated to the alkaline phosphatase enzyme was then applied (1:5,000 in PBS, Sigma) and incubated at RT for 20 min. After another washing step, the alkaline phosphatase substrate (p-nitrophenyl phosphate (pNPP), Sigma) was added and incubated for 10 to 30 min at RT until a yellow colour developed. The result was measured by spectrophotometry at 405 nm using a Titertek Multiscan MCC/340 (Labsystems) plate reader; a positive reaction confirmed that the protein had been correctly refolded. The coloured reagent was then washed off and the plate was probed with Streptavidin coupled to the enzyme horseradish peroxidase for 20 min at RT. The excess antibody was washed off; the enzyme substrate was added (O-phenylenediamine dihydrochloride, Sigma) and incubated for 10 to 15 min at RT. A yellow colour developed and the result was measured by spectrophotometry at 492 nm. A positive result confirmed that the HLA molecules had been correctly biotinylated.

Modified ELISA for chimeramer complexes

HLA-peptide biotinylated monomers used to prepare the chimeramer complexes were tested as described previously. Tetramerised Chimeramer complexes were also tested using a modified version of the previously described ELISA assay to confirm the presence of the antibody molecule in the complex.

The modification included involved a different capture antibody used in the first step that would confirm the presence of the co-stimulatory antibody in the complexes.

This capture antibody, being a goat anti-mouse antibody or a mouse anti-goat antibody at 5 µg/ml (both from sigma) was incubated on the Maxisorb immuno-plate (Nunc) as described previously. After incubating the samples to be tested, an additional step incubating diluted normal goat serum (100 µl of a 1:100 dilution per well, Sigma) was added to block any remaining free anti-goat antibody in the case of the mouse anti-goat ELISA first layer. This was necessary to prevent the goat anti-rabbit antibody conjugated to the alkaline phosphatase enzyme at the last layer from binding directly to the capture antibody (see

Figure II.11). The following steps were identical to the ELISA method described previously for the detection of correctly refolded HLA-peptide molecules.

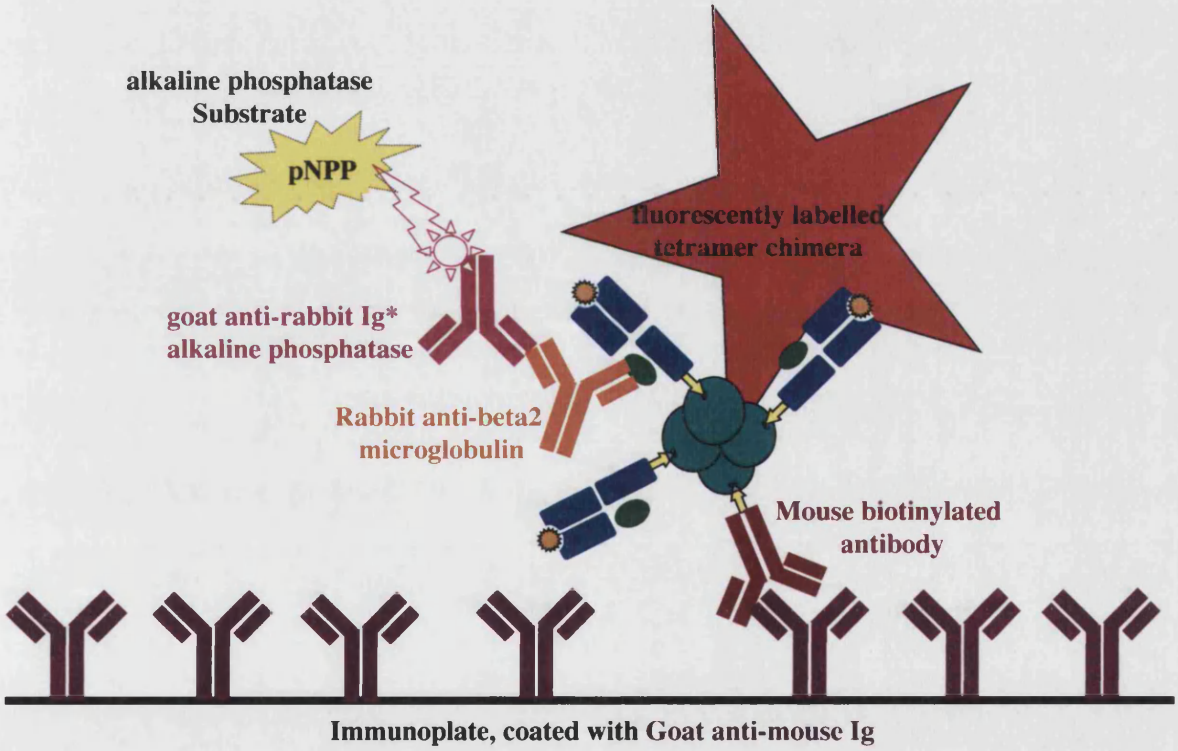

Figure II.11 Modified ELISA assay to test chimeramer complexes

HLA-peptide/antibody chimeramers are captured on an immunoplate pre-coated with a goat anti-mouse or mouse anti-goat (as applicable) antibody. The bound complexes are then probed with an anti-human β-2 microglobulin antibody to test for the presence of refolded HLA, and the reaction is developed.

IFN gamma ELIspot

The ELIspot assay consists in a modified version of the ELISA method, and allows the capture of proteins secreted by cells under specific culture settings. In the case of this study, the secretion of the cytokine interferon-γ (IFN-γ) by PBMCs upon peptide specific stimulation was measured. As the production of IFN-γ has an important role in T cell proliferation and activation, this was used as an in-vitro test for the functionality of these cells.

On the first day of the ELIspot assay, PBMCs were plated in round bottomed 96-well plates (BD) in two triplicate series of 100,000 cells or 50,000 cells per well. They were stimulated overnight with either PBS (negative and background release control), with PHA at 1 µg/ml (positive control) or with 10 µg/ml of antigen specific peptide and incubated overnight at 37°C in a water-saturated 5% CO_2 incubator. On the same day, a 96-well filtration plate (Multiscreen® Immobilon™-P filtration nitrocellulose plates, MAIP N45 10, Millipore) was coated with 15 µg/ml of monoclonal mouse anti-human IFN-γ antibody (1-D1K, ELIspot for human IFN-γ Kit, Mabtech) in PBS and incubated overnight at 4°C.

On the second day, any unbound anti-human IFN-γ antibody was washed from the Multiscreen plate extensively with sterile filtered PBS (0.45µm). The cells cultured the previous day were then transferred to this capture plate and incubated for 24 hrs at 37°C in a humidified 5% CO_2 incubator.

On the third day, the cells were removed from the capture plate. Each well of the Multiscreen plate was then washed six times with PBS. IFN-γ secreted by the cells following stimulation was captured by the monoclonal antibody and then detected by incubating with 1 µg/ml of biotinylated goat anti-mouse antibody (7-B6-1-biotin, ELIspot for human IFN-γ Kit, Mabtech) in filtered PBS with 0.5% (v/v) FCS. This was incubated for 2 hrs at RT and the plate was then washed six times with PBS. A 1:1,000 dilution of streptavidin-alkaline phosphatase (ELIspot for human IFN-γ Kit, Mabtech) in PBS with 5% FCS was then added and incubated for 1 hr at RT. After washing (6x PBS), the plate was finally incubated with 100 µl/well of alkaline phosphatase substrate (Alkaline phosphatase substrate package, composed of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine(BCIP), Gibco BRL) until dark blue spots emerged. The colour development was then stopped by washing with water, and after the plate was left to air-dry, the spots were enumerated under a

dissection microscope (x 20 magnification) and the samples were scored according to the number of spots counted per 100,000 or 50,000 cells.

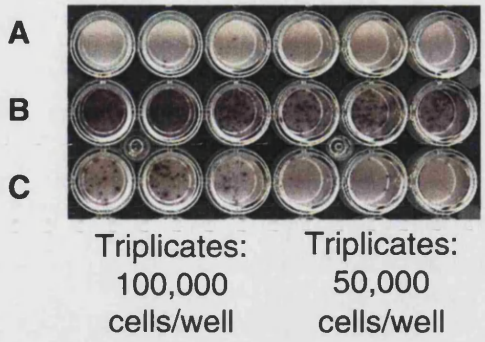

Figure II.12 IFN-γ ELIspot assay

Triplicate samples of 100,000 or 50,000 cells (in RPMI 1640 with 10% human AB serum) were incubated overnight with either: lane A, not stimulated (to control for the background release); lane B, stimulated with a potent mitogen (positive control: PHA at 1 µg/ml); or lane C, incubated with CMV peptide (here NLVPMVATV, 10 µg/ml). They were then transferred onto a sterile IFN-γ antibody coated plate and incubated for a further 24 hr. The cells were then discarded and the IFN-γ captured on the plate revealed. The number of spot forming units (sfu) was counted using a dissection microscope (x 20).

Magnetic bead selection

Tetramer positive T cells were purified after staining by magnetic separation. First, PBMCs were isolated from whole blood and specific staining with PE fluorescently labelled tetramer was performed as described previously. Labelled PBMCs were kept at 4°C during the whole purification procedure.

A two step purification protocol was used to first select $CD8^+$ T cells by mean of negative selection, then to purify the PE^+ cells by positive selection. The first step consisted in depleting B cells, monocytes, NK cells, T helper cells, dendritic cells, early erythroid cells, platelets and basophils from PBMCs resuspended in 80 µl of buffer (PBS, 0.5% bovine serum albumin and 2 mM EDTA, cooled at 4°C) by adding 20 µl of a cocktail of CD19, CD16, CD56, CD4, CD11b and CD36 antibodies coupled to an anti-hapten antibody per 10^7 total cells ($CD8^+$ T cell isolation Kit, Miltenyi Biotec). After 10 minutes of incubation, centrifugation and removal of the supernatant, 20 µl of Macs Microbeads were then added per 10^7 total cells. After a further 15 minutes of incubation, the cells were washed, resuspended in 500 µl of buffer (same as above) and loaded onto a MiniMacs column (Miltenyi Biotec) placed in the magnetic field of a

Macs separator. The cells labelled for depletion were then trapped in the column while $CD8^+$ T cells could be eluted freely from the column by gravity with the addition of buffer (same as above). Purified $CD8^+$ T cells were resuspended in 80 µl of buffer and then incubated with an anti-PE antibody coupled to an anti-hapten antibody in order to separate $CD8^+$ Tet^+ T cells from $CD8^+$ Tet^- T cells by positive selection. A similar procedure to that described previously was used to wash off any unbound reagent and add magnetic microbeads. The cells were then loaded onto a fresh MiniMacs column, and the fraction eluted with the buffer flow-through consisted of non-magnetically labelled $CD8^+$ Tet^- cells. After washing with several ml of buffer, the positive fraction of pure $CD8^+$ Tet^+ T cells were pushed through the column with a plunger into a tube containing 500 µl of warm RPMI culture medium for use in experiments.

Determination of Cytomegalovirus DNA viral load

The number of CMV genomes in the blood of patients was first measured by quantitative competitive PCR, then by an alternative method of real time PCR when the technique was updated in the Department of Virology at the RFH.

DNA extraction

Briefly, DNA was extracted from whole blood (200µL) using commercially available DNA extraction columns (Qiagen, Crawley, UK). All qualitative and quantitative PCR analyses were performed on 5µL aliquots (about 40 ng DNA, 6000 cell equivalent) derived from the 200 µL final whole blood DNA extract.

Quantitative-Competitive PCR method

A standard PCR reaction was set up with 1 unit of DNA polymerase Amplitaq Gold (Perkin Elmer, Warrington, UK), 1x PCR buffer II, 2mM $MgCl_2$, 100 ng each of glycoprotein B (gB) forward, gB1 and reverse, gB2 CMV primers (see Table II.17).

PRIMER SEQUENCES: CMV PCR	
gB 1 oligo primer (5'-3'): 25 mers	gB 2 oligo primer (5'-3'): 25 mers
GAGGACAACGAAATCCTGTTGGGCA	GTCGACGGTGGAGATACTGCTGAGG

Table II.17 glycoprotein B CMV specific primers

Forward and reverse glycoprotein B primers used to amplify CMV DNA by competitive-quantitative or real time PCR reactions.

The following program was then run:

Duration		Temperature (°C)	Effect
	12 min	95	denaturation
40 cycles	30 sec	94	denaturation
	30 sec	60	annealing
	30 sec	72	extension
	10 min	72	denaturation

Table II.18 CMV specific PCR

This table summarises all steps of the PCR reaction programme used to amplify double stranded gB CMV DNA isolated from patient samples.

A mutated internal control sequence of known copy number was included in each reaction, which can be differentiated from target amplicons after PCR

amplification by digestion with *Hpa*1 and separation on a 12% acrylamide gel. Gels were stained in ethidium bromide (0.15 µg/ml) and the viral load was calculated by comparing the intensity of the target band to the intensity of the control band using the NIH image analysis program. The data was expressed as $\log_{10}$ genomes/ml of whole blood.

Real time PCR method

Real time PCR reactions were performed in 96 well optical reaction plates (ABI Prism). The following components were added in the PCR mix (per tube): 1 unit of Hotstar Taq DNA polymerase (Qiagen), 1x PCR buffer, 2 mM $MgCl_2$, 100 ng of each of the gB primers (see Table II.17). Additionally, 100 ng of gB DNA probe labelled with the FAM-TAMRA fluorescent reporter complex (5(6)-Carboxyfluorescein or FAM; 5(6)-Carboxy-tetramethylrhodamine or TAMRA) were added. The specific probe annealed to the gB DNA between the forward gB1 and the reverse gB2 primers. Its sequence was as follows: 5'-**FAM**-CAATCATGCGTTTGAAGAGGTAGTCCACG-**TAMRA**-3').

As the PCR amplification occurs, the advance of the Taq polymerase exonuclease activity will induce the cleavage of the FAM-TAMRA complex, interrupting the transfer of energy to the fluorescent quencher TAMRA, allowing the TAM molecule to fluoresce. Therefore, the measured TAM fluorescence in the tube is directly proportional to the number of DNA copies amplified in each tube. The total volume of the PCR mix was 20 µl per well, to which 5 µl whole gB DNA positive control from 1 to 10^5 copies per well with 50 ng/µl of Salmon Sperm to facilitate amplification (Virology stock at 10^7 DNA copies/ 2 µl) was added. Similarly, 5 µl of negative control (50ng/ml Salmon sperm (Sigma) in Elution buffer (EB from the Qiagen DNA extraction kit) or EB alone), or 5 µl of patient DNA sample was added to 20 µl of PCR mix in the remaining wells. All positive control serial dilutions were tested in triplicate, a few negative controls were scattered throughout the plate, and all patient samples were tested in duplicate to ensure accurate and reproducible results. The Plate was then placed in a Taqman real time PCR machine 7700 sequence detector (ABI Prism), and the following program was run:

Duration		Temperature (°C)	Effect
2 min		50	Enzyme hot start
10 min		95	Denaturation
50 cycles	15 sec	95	Denaturation
	1 min	50	Annealing and extension

Table II.19 Real time Polymerase chain reaction

This table summarises all steps of the PCR reaction programme used to amplify gB cytomegalovirus DNA from patient samples with the 7700 sequence detector (ABI Prism).

The fluorescence in each well was measured 4 times every minute while the program was running. Data were analysed with the sequence detector v 1.7a software (ABI Prism) that plotted the level of fluorescence detected in each well as a function of the cycle number. This allowed to verify that the amplification had occurred, and to set a minimum threshold of detection. Negative controls were also checked.

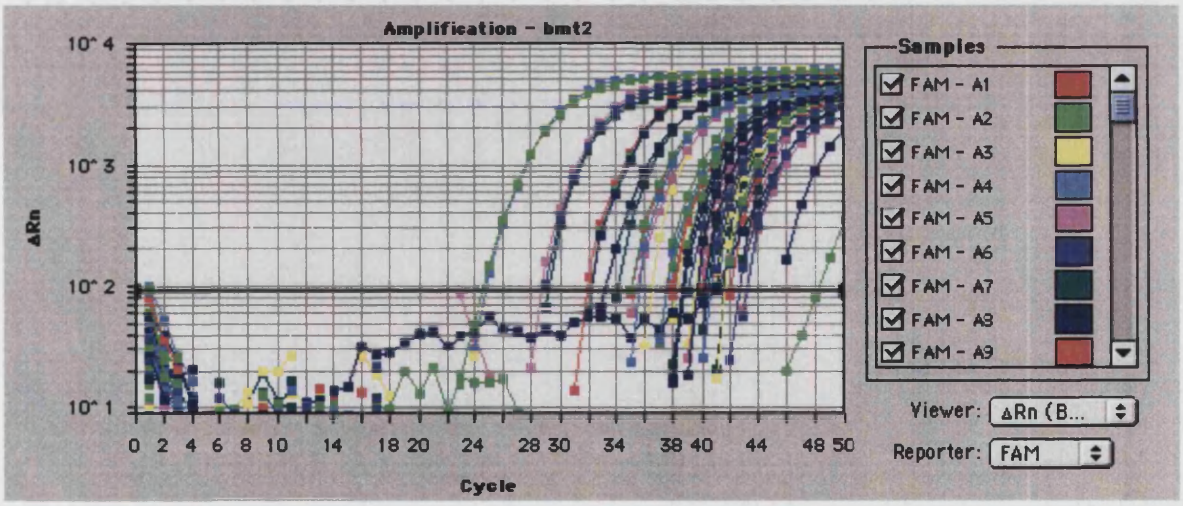

Figure II.13 Taqman PCR amplification for CMV viral load

The level of fluorescence (ΔRn) in each tube was plotted as a function of the cycle number and allowed to verify that each standard and sample had amplified correctly. It was also used to set the threshold of detection for the assay (—) so that the background fluorescence could be discounted from the analysis.

A standard curve was then produced and confirmed whether the reaction had worked with satisfying reliability (with a slope value close to the theoretical –3.5 and a correlation coefficient close to 1). The software assessed the amplification of the samples by determining the Ct value being the cycle number at which the PCR

amplification becomes detectable above the set threshold (typically the Ct value for one CMV copy number was 41 cycles).

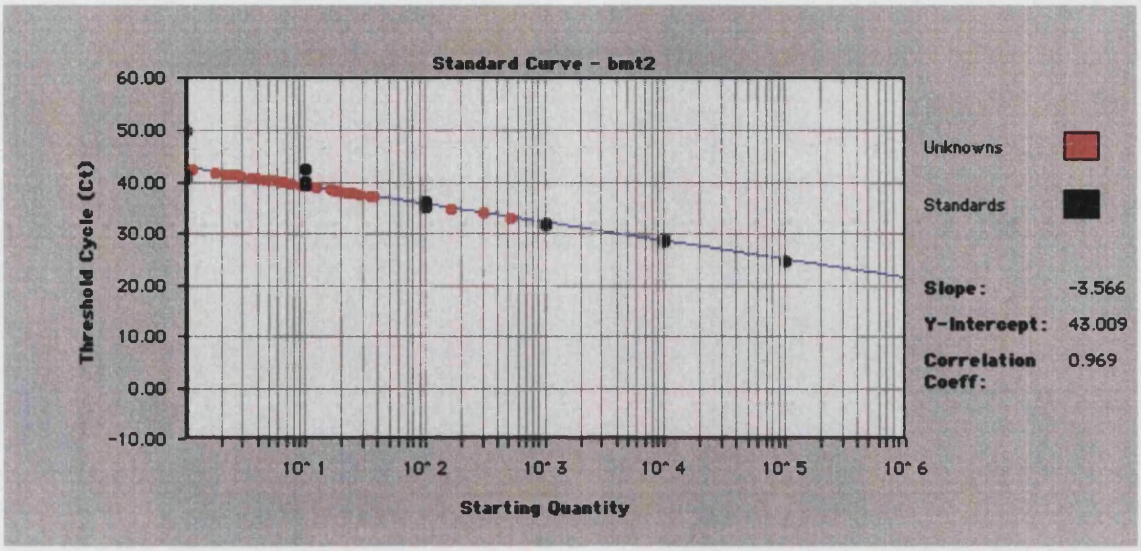

Figure II.14 Taqman PCR for CMV viral load: standard curve

A standard curve of the Ct results from the amplifications of the triplicate positive controls (●): from 1 to 10^5 copies of CMV DNA/ml of blood was plotted on a log scale graph. The slope at around –3.5 and a good correlation coefficient confirmed that the assay was reliable. This was used to plot the Ct of the samples tested (●) and to calculate their respective CMV copy numbers.

To calculate the number of CMV genomes in an unknown sample, the software placed the Ct of the sample on the standard curve and calculated the corresponding quantitative value.

The mean of duplicate values was taken for each 5 µl DNA sample tested and the result was expressed as $\log_{10}$ genomes/ml of whole blood.

Flow Cytometry

Flow cytometry allows the visualisation of single cell events and the simultaneous collection of a series of characteristics particular to that same single cell. It renders possible the collection of data characterising the size of the cell, its granularity and up to three or four cellular markers (in the case of the instruments available in the laboratory), labelled with fluorescent antibodies binding to cell surface molecules.

Instruments

Flow cytometric acquisition of stained cellular samples was performed on the Facscan Instrument, which comprised an argon-ion laser for three colours fluorescence detection. The instrument was upgraded to a Facscalibur Instrument, which was composed of both an argon ion laser and a red diode laser in early 2002 that allowed four colours fluorescence detection). Both instruments were purchased from Becton Dickinson, who calibrated and maintained both instruments.

Acquisition and/or Analysis

Acquisition and analysis on the flow cytometers were performed using the CellQuest version 3.3 software (BD). This was used to visualise the cellular events as they were being acquired by the instrument, to set the compensation (and time delay calibration for four colour calibration on Facscalibur) for the fluorescence detection and later, to analyse the samples.

Statistical analyses

The statistical tests used to assess the data reported in the following chapters were carried out using the Prism 3 Software (GraphPad) package.

Chapter III
Detection of Antigen Specific Immune Responses

Introduction

Prior to the discovery of HLA/peptide tetramer complexes, the standard methods for detection and quantification of antigen specific T lymphocyte responses were based on the proliferative capacity of these cells (limiting dilution analysis) or their activation and response following antigen specific stimulation and assessment by interferon-γ ELIspot. Although these tests constitute good markers of activation and effector function, their drawback resides in the fact that all cells specific for the same antigen are unlikely to give a 100% response to an *in vitro* (and perhaps sub-optimal) stimulus and proliferate at a given time. Therefore these techniques have the disadvantage of underestimating the true number of antigen specific T cells present in peripheral blood.

The first report of the use of HLA/peptide tetramer complexes in 1996 by Altman *et al* revolutionised the field of immunology by describing a new method that allowed the detection of antigen specific T lymphocytes specifically using their receptor specificity. This was achieved by detecting T lymphocytes with a fluorescent complex of their specific natural ligand directed at the T cell receptor: the HLA/peptide molecular complex. The fast dissociation rate of the HLA/peptide molecule from the TCR was compensated for by the formation of a tetramer of HLA/peptide molecules, which resulted in an increased avidity of the complex for the TCR. This permitted the detection and phenotyping of antigen specific T lymphocytes by flow cytometry. This method is described in further detail in Chapter II.

At the start of the work described in this thesis in 1999, this was the main reference describing the synthesis of HLA/peptide tetramer complexes. They had been used successfully for the detection of specific $CD8^+$ T cells in the context of several human viral infections: human immunodeficiency virus (Altman *et al*, 1996; Ogg *et al*, 1998), influenza (Dunbar *et al*, 1998) and Epstein-Barr virus (Callan *et al*, 1998).

As these complexes were not available commercially at the time, the first objective of this PhD project was to set up a reliable method to make HLA/peptide tetramer complexes at the ANRI to enable the application of the most sensitive method to date for studying peptide specific cytotoxic T cell responses. The focus of this chapter is to outline the steps that were taken to apply this technology and to detect antigen specific T cell responses of relevance to the treatment of haematological malignancies and viral pathology occurring in patients receiving SCT.

CMV

The CMV study was initially focused on the HLA-A*0201 molecule, because it is the most common HLA subtype in many ethnic groups, with an antigen frequency of up to 42.8% in the Caucasoid population (Marsh *et al*, 2000). Hence, it was chosen for cloning and preparing HLA/peptide tetramers in order to maximise the number of individuals that could be tested.

In SCT recipients, the CTL component of the immune response to CMV was shown to be essential for their recovery from viral reactivation (Quinnan *et al*, 1982, 1984; Borysiewicz, 1988; Riddell *et al*, 1991). Furthermore, the adoptive transfer of CD8 specific T cell clones and the transfusion of leucocytes was used successfully and proven to be sufficient to resolve CMV reactivation post transplantation (Walter *et al*, 1995; Riddell *et al*, 1997; Witt *et al*, 1998). The antigenic targets for the CTL component of the immune response were demonstrated by several research groups and were mainly directed against the following proteins: IE-1 (or pp72, UL123), pp150 (UL32), pp65 (UL83) and gB (UL55) (Borysiewicz *et al*, 1988; Wills *et al*, 1996; Kern *et al*, 1999; Gyulai *et al*, 2000; for a review on potential vaccination targets, see Plotkin, 2002). Although the total CTL response has several components directed towards a range of targets, the pp65 protein was found to be a major target and shown to be immuno-dominant in the context of HLA-A*0201 (McLaughlin-Taylor et al, 1994; Wills et al, 1996; Diamond et al, 1997).

Previous work at the ANRI defined seventeen CMV pp65 candidate peptides selected using a computer algorithm, which tested for the presence of HLA-A*0201 specific anchoring residues and for potential binding capacity of these peptides to HLA-A*0201. Subsequently, actual binding affinity was shown with both a T2 binding assay (or ability of the pulsed peptide to stabilise HLA expression at the surface of T2 cells) and a peptide competitive binding assay (competitive binding of the test CMV peptide to displace and bind in place of a control peptide, which was used to give 100%

binding) by Solache *et al* (1999). Three of these 17 peptides, AE42, AE44 and AE45 (see Chapter II) could bind HLA-A*0201 molecules with high or intermediate affinity. These peptides were used to stimulate T cells: the cells were stimulated twice with peptide pulsed autologous PHA blasts, followed by two rounds of stimulation with peptide pulsed T2 cells to raise CMV specific lines. Only the cell lines generated against AE42, AE44 and AE45 could kill peptide pulsed T2 targets, CMV infected cells and cells infected with an adenoviral construct coding for the pp65 antigen. None of the other peptide tested generated any CMV specific T cells after stimulation.

The fact that CMV reactivation remains a major concern post SCT was highlighted in Chapter I. Prior to embarking on the study of CMV specific immune recovery in HLA-A*0201 positive SCT recipients, it was first necessary to establish that CMV specific CTL responses could be reliably detected in samples from healthy CMV seropositive donors and from SCT recipients using the tetramer technology.

Based on these results, HLA-A*0201/peptide complexes specific for these three CMV peptides were generated to test whether CMV seropositive individuals possessed peripheral blood T cells that are specific for any of these HLA/peptide combinations. This would confirm whether these peptides are processed, and presented on the surface of CMV infected cells and are able to elicit a specific T cell response. The synthesised complexes detecting a positive response would then be used for the detection, quantification and characterisation of CMV specific responses.

CML

The success of donor leucocyte infusions for CML patients with relapsed disease after SCT triggered the search for the immunological component of the response that was observed clinically. A number of potential targets for an immune response were envisaged: mismatched minor histocompatibility antigens, leukaemia reactive antigens that are highly expressed in leukaemia positive cells but also present in normal cells, and leukaemia specific antigens that only exist in leukaemia positive cells (for a review, see Falkenburg *et al*, 1997).

As part of a collaborative study between three research centres (ANRI, Nottingham Trent University and University of Liverpool) we set out to determine whether leukaemia specific T cells could be identified in the blood of CML patients, thus helping to resolve the question of the potential leukaemia specific immunogenicity of CML cells. Focus was put on the BCR/ABL b3a2 leukaemia specific fusion protein in HLA-A*0301 positive patients. This combination was chosen for investigating the

presence of fusion peptide specific T cells in patients and their immune potential, as a number of reports had previously demonstrated the intermediate affinity of the KQSSKALQR peptide spanning the b3a2 BCR/ABL fusion region for the HLA-A*0301 molecule (Bocchia *et al*, 1995; Buzyn *et al*, 1997; Berke *et al*, 2000). Additionally, a retrospective analysis of the HLA allele frequencies observed in CML patients revealed that HLA-A3 and particularly HLA-A3 associated with HLA-B8 were linked with a reduced risk of developing CML malignancy. Furthermore, the KQSSKALQR peptide was shown to be able to induce HLA-A*0301 restricted *in vitro* cytotoxic activity in PBMC samples from healthy donors and from patients, which suggested that triggering of anti-leukaemia specific T cells was a distinct possibility (Bocchia *et al*, 1996; Norbury *et al*, 2000).

PBMCs were isolated from blood samples obtained from CML patients and were tested for the presence of HLA-A*0301/KQSSKALQR specific T cells. HLA-A*0301/peptide tetramer complexes prepared with the KQSSKALQR peptide (or HLA-A*0301/CML tetramer) were used to determine whether CML specific T cells could be detected in the peripheral blood of HLA-A*0301 CML patients. They were prepared with the assistance of Audrey Bourdon, an undergraduate student at the ANRI.

Results

CMV specific immune T cell responses

Flow cytometry analysis of HLA/tetramer stainings

HLA/peptide tetramer complexes were composed of fluorescently labelled streptavidin molecule that allowed their detection (see Chapter II). Following the binding of tetramers to their specific TCR ligand on the T cell surface and removal of any unbound reagent by washing, the fluorescently marked cells were then detected by flow cytometry.

After isolating PBMCs from freshly drawn blood, they were first stained with HLA/peptide tetramer, followed by stainings with two CD markers. The first was the CD3 marker specifically binding to T cells, and the second was the CD8 marker specifically binding to the cytotoxic T cell subset. These were visualised using a Facscan instrument, which allowed the simultaneous detection of 3 fluorescent colours. Sequential gating of $CD3^+$ T cells (panels A and B, Figure III.1), then of $CD3^+CD8^+$ T cells (panel C) was used to ensure the specificity of the cytotoxic T cell population of

interest. This gating strategy was necessary for the accurate detection of tetramer positive (Tet^+) T cells, for monocytes as well as apoptotic or necrotic cells can also bind tetramer reagents in a non specific fashion and need to be discounted from the analysis. The quantification of tetramer positive cells in the samples was expressed as a percentage of $CD8^+$ T cells (panel E, as reported in most references using the tetramer methodology), or as a percentage of $CD3^+$ T cells (panel D). This facilitated the subsequent calculation of the absolute number of Tet^+ T cells per ml of blood. In addition, this gating strategy proved to be useful with tests on SCT recipient samples, particularly at early times post transplant, or when cytotoxic drugs were administered to the patient when analysis could be difficult due to low cell number and/or cell lysis.

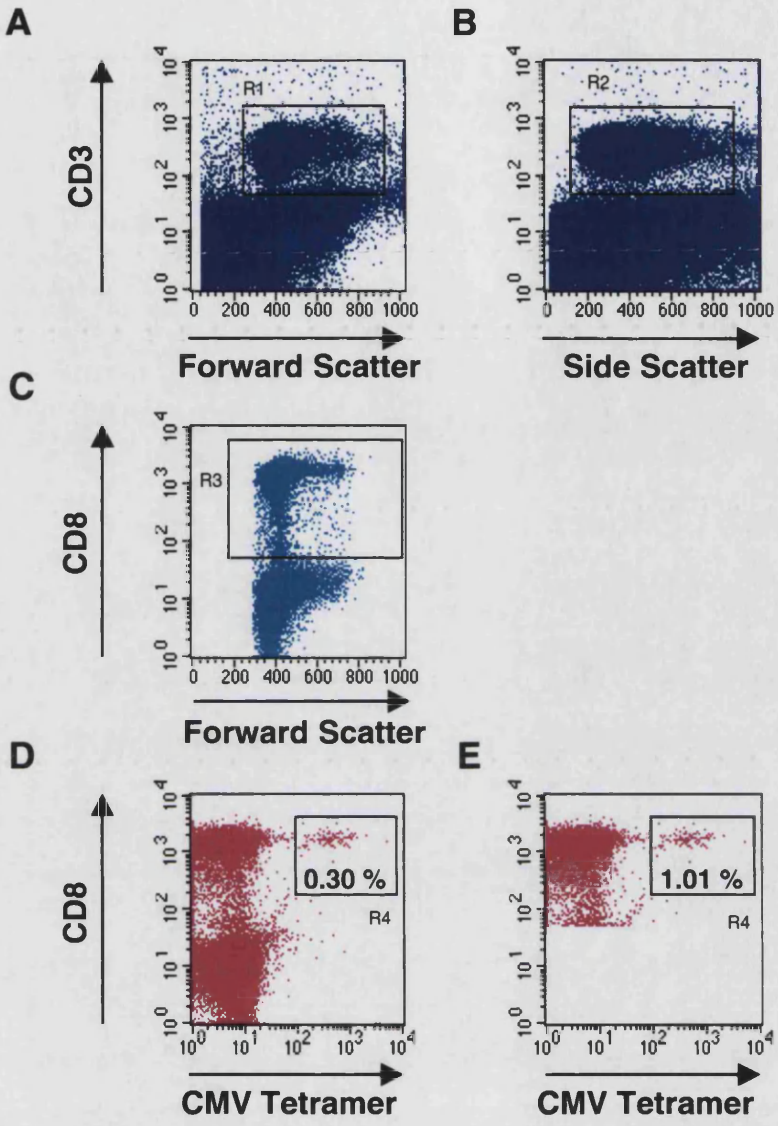

Figure III.1 Tetramer analysis and gating strategy

Triple staining of an HLA-A*0201 CMV$^+$ healthy individual with HLA-A*0201/AE42 CMV specific tetramer, CD3 and CD8 antibodies are represented. All detection experiments were performed in a similar manner. First CD3$^+$ T cells were gated on the forward and side scatter panels (A and B), then the CD8$^+$ fraction of these CD3$^+$ cells were gated (panel C). The tetramer positive events were gated thereafter and their numbers expressed as a percentage value of CD3$^+$ or CD3$^+$CD8$^+$ T cells as indicated on panel D and panel E respectively.

Determination of CMV pp65 antigenic peptide targets

A preliminary study assessing the CMV specific CTL responses of a selection of healthy individuals and SCT recipients was conducted. The three CMV peptides previously discussed were tested to determine which ones would be relevant markers

for CMV specific CTL responses. Therefore CMV specific tetramer complexes were prepared with these three pp65 peptides (AE42, AE44 and AE45) and tested to answer this question. PBMC stainings and analyses were performed as indicated in the previous paragraph, with representative results shown in Table III.1 and illustrated in Figure III.2. Additionally, HLA-A*0201/HA-1 minor antigen specific tetramer reagents were used in some cases as an additional negative reagent control.

			HLA-A*0201 Tetramer staining, %age of $CD8^+$			
Sample	HLA type	CMV[b]	AE42	AE44	AE45	HA-1
HD[a] 1	A*0201	+	0.60	0.02	0.03	0.02
HD 2	A*0201	-	0.04	0.01	001	nd
HD 3	A*30/A*31	+	0.02	0.01	0.02	nd
Patient 4	A*0201	+/+	11.17	0.02	0.02	0.01
Patient 9	A*0201	-/-	0.02	0.01	0.03	0

Table III.1 Results of representative CMV specific tetramer stainings

[a] HD, healthy donor; [b] CMV, cytomegalovirus serostatus and patient/donor serostatus when applicable.
Responses to three CMV epitopes (AE42, AE44 and AE45) were measured by HLA-A*0201/peptide tetramer staining. T cells specific for the HLA-A*0201/AE42 tetramer were only detected in CMV seropositive individuals as seen for HD 1 and Patient 4. HD 1 is representative of 8/9 healthy CMV^+ donors, Patients 4 and 9 are representative of 12 SCT recipients tested over a 4 months period. HD 2 is representative of 5 CMV^- healthy donors tested.

As can be deduced from the representative samples reported in Table III.1, none of the individuals tested showed CTL populations specific for the HLA-A*0201/AE44 or the HLA-A*0201/AE45 tetramers. Neither the $HLA\text{-}A*0201^-$ CMV^+ (one healthy individual tested), nor the $HLA\text{-}A*0201^+$ CMV^- samples tested (representative of 5 healthy individuals and 5 SCT recipients tested) stained with any of the tetramer reagents. The HLA-A*0201/AE42 tetramer was the only one that stained consistently showing a discrete population of CTLs in all but one of the HLA-A*0201 CMV seropositive individuals. The sample from donor HD 1 (0.60% of $CD8^+$ T cells) is representative of 8 healthy individuals tested, and the sample from Patient 4 (11.17% of $CD8^+$ T cells) is representative of 12 SCT recipients tested in this preliminary study. The only healthy CMV^+ volunteer that did not have any HLA-A*0201/AE42 specific T cells was later shown to respond to the CMV IE-1 antigen by another student in the

laboratory. The patients were therefore tested in a longitudinal study to determine if CMV reactivation would trigger the emergence of HLA-A*0201/AE44 or HLA-A*0201/AE45 specific T cells. All tetramer stainings performed in this context were negative. Although no HLA-A*0201/AE44 nor HLA-A*0201/AE45 specific T cells could be detected in samples from healthy individuals or SCT patients, there was still a possibility that these cells may be present at a level below the detection limit of the assay. A specific antigenic stimulation should result in the proliferation of these cells, which should bring them above the detection limit. CTL responses to HLA-A*0201/AE42, HLA-A*0201/AE44 and HLA-A*0201/AE45 were examined in the context of CMV reactivation after SCT. The result obtained from representative samples of a CMV^+ SCT recipient (patient 4) are represented on Figure III.2, together with a sample from a healthy CMV^+ donor sample. The data presented clearly demonstrate the fact that only T cells specific for the HLA-A*0201/AE42 combination proliferated in response to an "*in vivo*" CMV antigenic stimulus that could be detected by tetramer staining. By contrast, no T cells specific for HLA-A*0201/AE44 or HLA-A*0201/AE45 could be seen above the detection level of the assay. This further confirmed that no T cells specific for HLA-A*0201/AE44 or HLA-A*0201/AE45 are present in the peripheral blood from CMV^+ healthy individuals or SCT recipients.

The HLA-A*0201/AE42 was therefore chosen for the identification of CMV pp65 specific T cells in PBMC samples. This tetramer complex will be referred to as CMV tetramer in the following paragraphs.

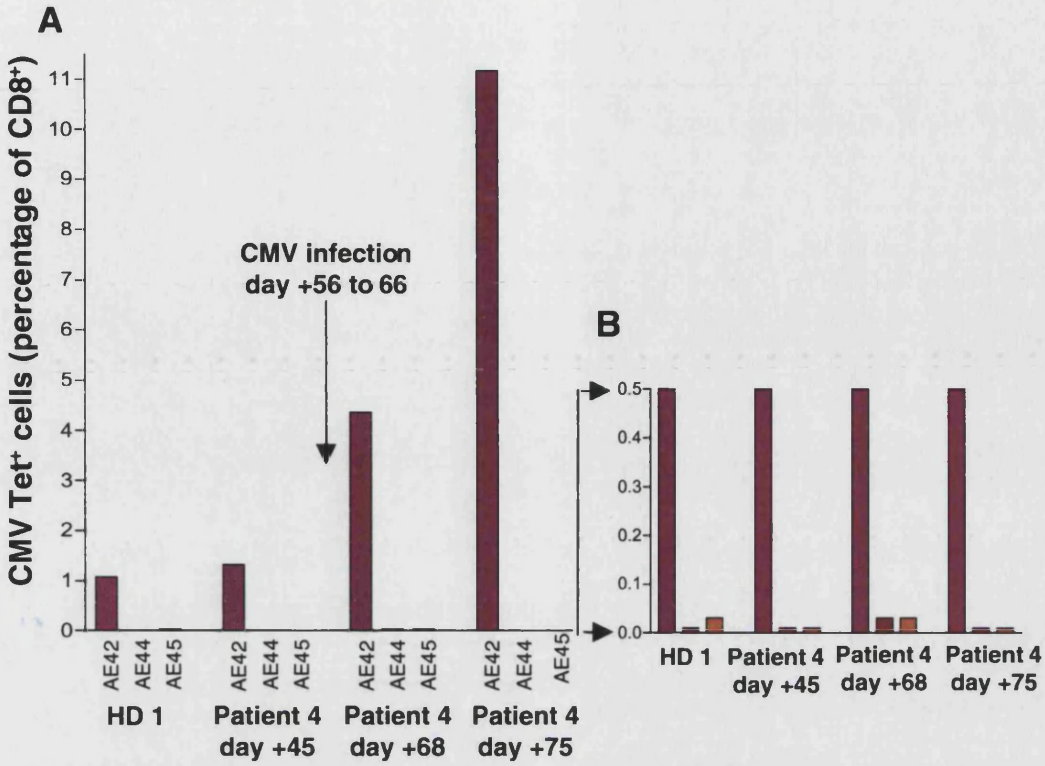

Figure III.2 CMV tetramer specific staining

Representative stainings with HLA/peptide CMV specific tetramer reagents prepared with the peptides: AE42 (■), AE44 (■) and AE45 (■). 4 individual samples are shown: one from a healthy CMV^+ donor (HD1) and 3 from a CMV^+ recipient of a CMV^+ graft (patient 4) before and after occurrence of CMV infection after SCT (panel A), with the section from 0 to 0.5% $CD8^+Tet^+$ cells magnified (panel B).

CMV tetramer staining specificity

Staining temperature

The issue of staining specificity for tetramers is essential for the accurate detection of antigen specific T cells. Whelan et al (1999) demonstrated that the specificity of tetramer staining was temperature dependent and that the highest specificity was obtained by staining at 37°C, with some degree of cross reactivity and that additional non specific staining was observed at lower temperatures. Tests on the staining specificity of CMV tetramers were performed on samples from healthy CMV seropositive individuals at both 4°C and 37°C. Results were concordant with the data generated by Whelan et al (data not shown). Accordingly, and as mentioned in Chapter II, all subsequent HLA/peptide tetramer stainings were performed at 37°C for 30 minutes.

Competitive block of CD3 binding

As described previously, HLA/peptide tetramer complexes bind to their natural ligand on the T cell: the TCR. The CD3 molecule is part of the TCR complex and therefore lies in close proximity to the α and β chains of the TCR molecules on the cell surface. As a consequence, the binding of tetramer complexes to the α/β TCR might prevent to some degree the subsequent staining with anti-CD3 antibodies. However, non-specific and/or low affinity binding of the tetramer to its ligand would not prevent the binding of the anti-CD3 antibody. This phenomenon was confirmed by Hoffmann *et al* (2000) and can be used as a test of specificity for the binding of HLA tetramers to their respective TCR. This can be visualised by comparing the geometric mean of CD3 fluorescence intensity in the lymphocyte population, to the geometric mean CD3 fluorescence intensity in the $CD8^+Tet^+$ cell population. The ligand specificity is confirmed if the level of CD3 antigen visualised on the Tet^+ T cell surface is lower as compared to the expression on the general T lymphocyte population.

This procedure was applied to CMV specific stainings (see Figure III.3), which were characterised by high affinity of the peptide for the HLA-A*0201 molecule and relatively high T cell frequencies in peripheral blood. Figure III.3 shows two representative samples from HD1 and patient 6 respectively, where live lymphocytes were gated, as well as $CD3^+$ cells and the $CD8^+$ Tet^+ cells subsequently visualised.

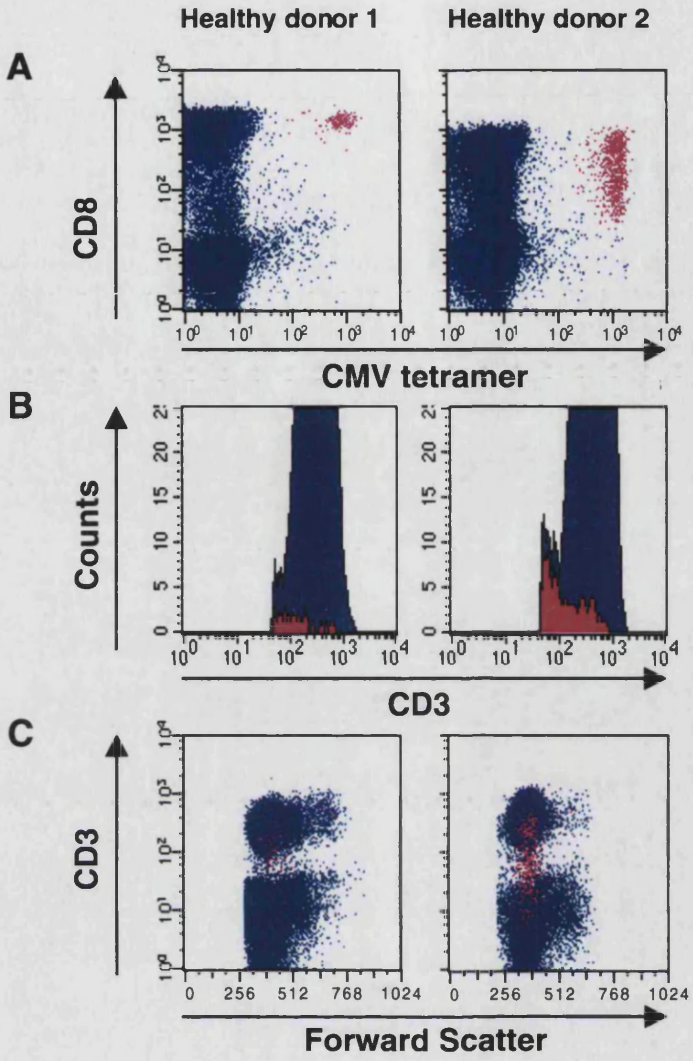

Figure III.3 Specificity of tetramer staining

Competitive block of CD3 antibody binding following tetramer staining.
Two healthy donors are represented. Live lymphocytes were gated (panel A, ●), and $CD8^{+}Tet^{+}$ cells painted in pink (●). A histogram plot of CD3 staining intensity is shown on panel B with the gated $CD3^{+}Tet^{+}$ population in pink. The respective geometric mean fluorescence intensity values of the CD3 marker were: HD 1, $CD3^{+}$: 306, $CD3^{+}Tet^{+}$: 101; patient 6, $CD3^{+}$: 403, $CD3^{+}Tet^{+}$: 106. This can also be visualised on the dot plot (panel C) representing the gated live lymphocyte populations using Paint-a-Gate, and the CD3 expression of the whole CD3 T cell population (top half) and the $CD3^{+}Tet^{+}$ T cell population in pink.

The mean fluorescence intensity of the CD3 marker between the $CD3^+$ Tet^- T cell population and the $CD3^+Tet^+$ population was measured. This level of fluorescence was consistently and significantly lower in the T cells that bound the tetramer reagent prior to the incubation with the CD3 antibody, therefore indicating the specific occupancy of the αβ TCR on Tet^+ cells (CD3 fluorescence intensities of: 101 versus 306 and 106 versus 403 for HD1 and patient 6 respectively). It proved to be a reliable way to check the specificity of tetramer stainings and also particularly useful in testing the consistency of the stainings when new batches of tetramer reagents were prepared.

However, this assay may not be suitable for testing all HLA/peptide tetramer reagents as Hoffmann *et al* noted that this test had proven inconclusive in the case of rare tetramer positive events.

Correlation of CMV tetramer staining with CMV specific effector function

The release of IFN-γ following specific T cells activation has been shown to be important in the fight against viral pathogens with its direct anti-viral properties involving the prevention of viral replication in previously uninfected cells as well as immunoregulatory effects (Guidotti and Chisari, 1996; Biron *et al*, 1999, 2001; Durbin *et al*, 2000). The other major functions of IFN-γ are the recruitment of macrophages to the site of infection, their activation, and the up-regulation of the expression of MHC molecules and antigen processing components in cells upon binding of the IFN-γ receptor.

The production of IFN-γ following peptide stimulation of freshly isolated PBMCs from HLA-A*0201 and CMV^+ healthy individuals was examined by ELIspot assay. In parallel, CMV tetramer staining was performed to determine the frequency of CMV peptide specific T cells. The two analyses were run simultaneously on fresh PBMC samples, and the same CMV pp65 specific peptide was used for both techniques. The results obtained for three representative healthy CMV^+ individuals of six tested are represented on Figure III.4 and were compared with the Pearson's test to establish whether the two correlated. The correlation between the two data sets was calculated and the resulting Pearson's coefficient was $r=0.99$ ($p<0.001$). This result represented a high correlation between CMV specific tetramer staining and IFN-γ that was consistent with the concept that the CMV Tet^+ CTLs were capable of mediating an antiviral response in CMV^+ healthy individuals.

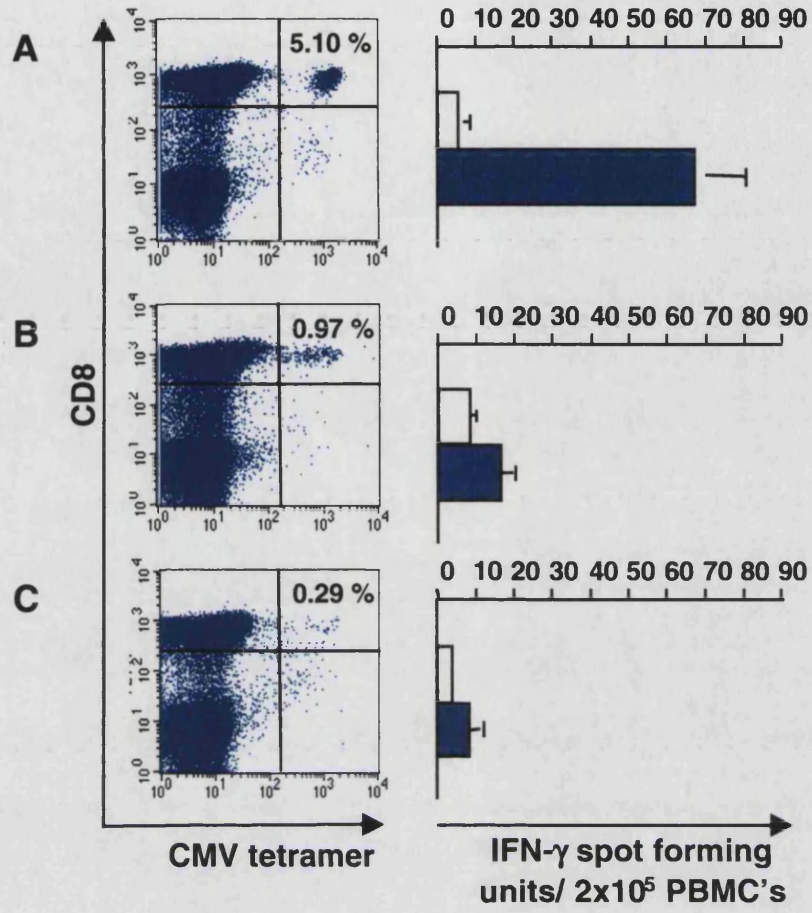

Figure III.4 CMV peptide specific effector function

CMV tetramer staining and CMV peptide specific $CD8^+$ T cell IFN-γ ELIspot assays were performed in parallel on freshly isolated PBMC samples from healthy CMV^+ individuals. CMV Tet^+ T cell frequencies are indicated on the dot plots and are expressed as a percentage of $CD8^+$ T cells. The number of spot forming units (sfu) per $2x10^5$ PBMCs after overnight peptide stimulation (AE42 at 10 µg/ml, ■) in ELIspot assays were counted; the background IFN-γ release (medium alone control, □) was subtracted before the degree of correlation between the two measurements was assessed.

other available option was to use a T cell clone, which would provide good purity of the cellular population to be tested. As T cell clones specific for CMV were known to be available, colleagues in Leiden were therefore contacted, and they kindly agreed to donate a cryopreserved HLA-A*0201/CMV AE42 restricted T cell clone.

An assay measuring the rate of apoptosis (or programmed cell death) by staining for intracellular caspases (Apostat) was chosen and was set up rather than using the more classic chromium (Cr^{51}) release assay because T cell numbers were limiting. All pathways inducing apoptosis lead to the activation of a series of caspase enzymes that end with the activation of a DNase that transfers to the cell nucleus and cleaves DNA therefore causing cell death. Substrate recognition motifs are conserved between caspases and were exploited to develop an irreversible competitive fluorescent peptide that can then be detected in cells undergoing apoptosis. This technique was chosen in order to reduce the number of cells from the clone used in each experiment. CMV pulsed T2 target cells were labelled with the membrane red dye PKH 26 and incubated with differing ratios of CMV specific T cells; after 3 hours they were then assessed for apoptosis with the Apostat staining reagent (measuring the level of active caspase enzymes labelled with the FITC marked peptide). A major problem that was encountered in these experiments was that the CMV specific T cell clone lost viability rapidly and cultures could not be maintained with over 50% viability. Attempts to recover viable cells by density gradient centrifugation were unsuccessful. Staining with anti-CD95 antibody revealed a high expression level on the surface of Tet^+ cells, suggesting a high apoptotic potential for these cells. This resulted in disappointing results for the killing assay that could not be reproduced reliably after three attempts following short-term *in vitro* culture with IL7 and IL15.

Despite this unsatisfactory outcome, specific killing was observed using the same effector and target system (but with a different labelling combination) by fluorescent microscopy as seen in Figure III.5. Binding of the tetramer labelled T cells to the larger peptide pulsed T2 target cells was observed (Figure III.5, C). This demonstrated the specific targeting of pulsed T2 cells by the T cell clone and subsequent cytotoxic killing as visualised by the apoptotic morphology of the target (irregular shape, shrinking cytosol and presence of large granules, Figure III.5,D).

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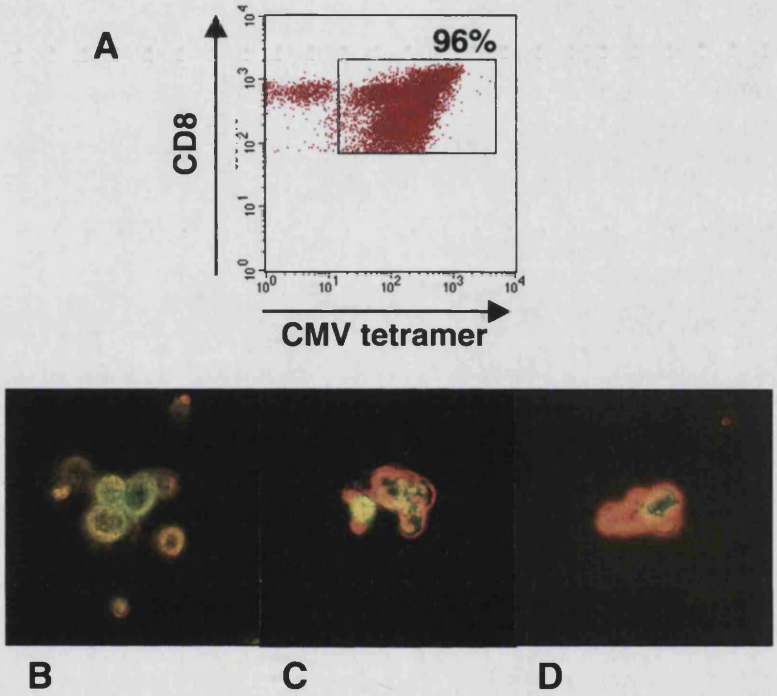


Figure III.5 CMV Tetramer$^+$ T cell cytotoxicity

A T cell clone specific for CMV was purified from a HLA-A*0201 healthy donor, was generated by single cell sorting and subsequent *in vitro* expansion. This clone was stained with HLA-A*0201/CMV tetramer and proved to be 96% pure (panel A). T2 cells were labelled with the green PKH 67 dye, washed and pulsed with 1 mg/ml CMV AE42 peptide for two hours (panel B). Cloned T cells pre-stained with tetramer were added to the T2 at a 1:1 ratio and incubated at 37°C. 15 minutes after mixing the two cell types, attachment of the T cells to the T2 targets was observed (panel C). The T cells could later be observed still attached to a target, which presents typical apoptotic morphology after half an hour to an hour (Panel D).

The synthesis and appropriate testing of HLA/peptide tetramers allowed the detection of antigen specific CD8 T cells in healthy individuals. Positive tetramer staining could be correlated with effector function upon peptide stimulation and together with target specific cytotoxicity. The HLA/peptide tetramer technology was therefore applied to the detection of antigen specific responses of potential diagnostic

and/or therapeutic interest for the treatment of patients with haematological malignancies.

CMV specific T cell detection in patients post SCT

Following the generation of HLA-A*0201 restricted tetramers containing the immunodominant peptide AE42 from CMV pp65 and the identification a tetramer positive population of $CD8^+$ T cells in healthy seropositive individuals, SCT samples were obtained after engraftment and were tested for the presence of CMV specific T cells.

Patients characteristics are described in Chapter II, in particular the CMV serostatus of the patients and that of their donors, which were relevant to the detection of CMV specific T cells. It had been previously established (Table III.1 and Figure III.2) that only HLA-A*0201 restricted T cell responses to the AE42 CMV peptide were detectable in both healthy donors and SCT recipients. All 19 CMV^+ SCT recipients showed detectable populations of CMV tetramer specific T cells whether they were expected to have acquired specific T cell immunity from the graft they received (for the patients with CMV^+ donors) or not for the CMV^+ patients with CMV^- donors who subsequently had CMV infection following transplant. Representative samples are shown on Figure III.6, with HD1 a healthy CMV^+ control. As is the case for healthy CMV^+ donors, CMV specific T cells can be reliably detected in PBMC samples from CMV^+ patients after SCT using HLA/peptide tetramer reagents. Additionally, CMV specific T cells were successfully detected after staining with HLA/peptide tetramer reagent in all CMV^+ recipients, whether their donor was CMV^+ or CMV^-. Therefore patients with CMV^- donors, although having no transferred memory T cell immunity, should be able to mount a cytotoxic response to CMV challenge (Figure III.6, patient 1). It can be noted that CMV^- patients with CMV^- donors consistently had negative staining with CMV Tet, highlighting an absence of CMV specific T cell immunity in these patients and giving further evidence to confirm that these patients had not previously contracted CMV. This allowed for the reliable detection and longitudinal study of CMV specific T cells in all SCT patients at "risk" of cytomegalovirus reactivation and CMV disease post SCT ($CMV^{+/-}$ and $CMV^{+/+}$). The CMV specific immune recovery of all patients recruited in the CMV study will be further examined later in Chapter IV.

The accurate identification of CMV specific Tet^+ T cells can be exploited to further characterise these cells and their properties.

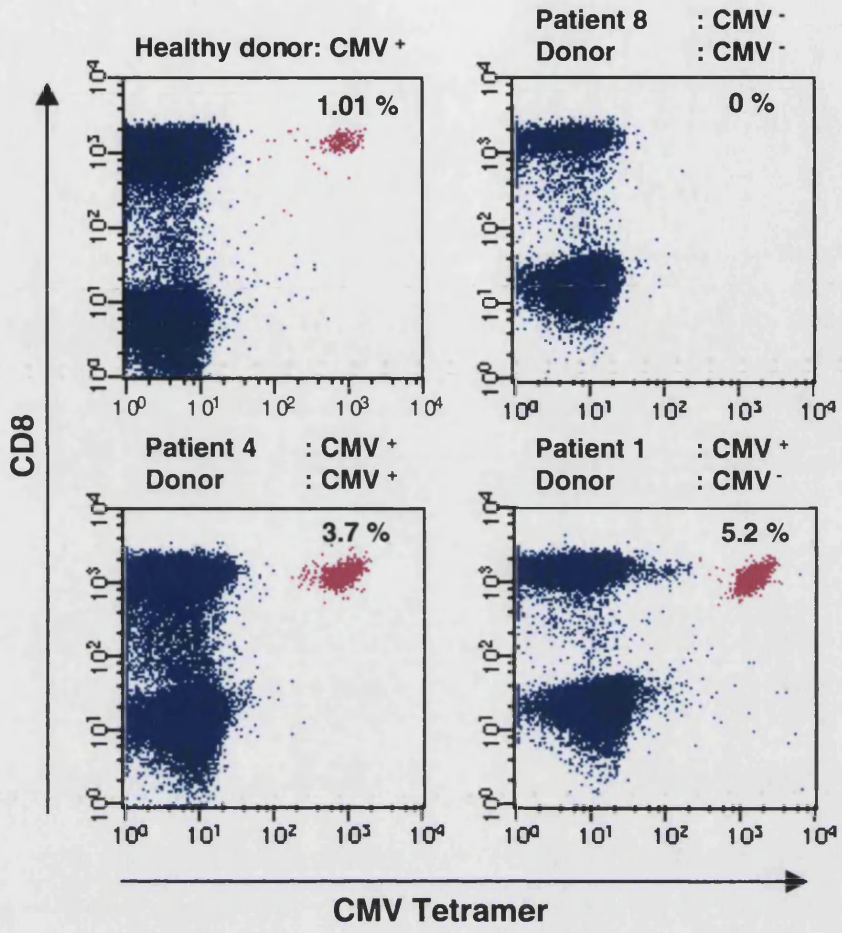


Figure III.6 Detection of CMV specific T cells in SCT recipients

Representative CMV tetramer stainings, gating on $CD3^+$ T cells (blue), and representing $CD3^+CD8^+$ T cells in the upper half of each panel, with $CD3^+CD8^+Tet^+$ T cells in pink. As shown in CMV^- healthy individuals, no Tet^+ T cells were detected in samples from CMV^- recipients of SCT with a CMV^- donor. As shown for CMV^+ healthy individuals (Healthy donor panel), Tet^+ discrete T cell populations were detected in all CMV^+ SCT recipients, regardless of their donors' serostatus (CMV^+,Patient 4; CMV^-,Patient 1).

Phenotypic analysis of CMV specific CD8 T cells

Further phenotypic analysis of CMV specific Tet^+ CD8 T cells became possible in the later stages of these studies with the acquisition of a Facscalibur flow cytometry instrument allowing the simultaneous detection of four distinct fluorescent colour emissions, by simultaneously staining for tetramer and antibodies to specific cellular molecules that are characteristic for distinct cell populations.

Traditionally, the two distinct isoforms of the transmembrane phosphatase CD45 were used together with CD8 to separate naïve cytotoxic T cells $CD45RA^+$, from antigen experienced (or memory) $CD45RO^+$, as the latter form becomes up-regulated

after activation (Akbar *et al*, 1988; Merkenschlager and Beverley, 1989). However this concept has since been re-evaluated as CD45RA high populations were shown to comprise both naïve and memory T cell subsets (Wills *et al*, 1999), and these CD45RA high memory T cells were thought to constitute a terminally differentiated T cell population or terminal effectors. To characterise tetramer positive T cell populations in SCT recipients in whom these responses are likely to be originating from donor T cells transfused with the graft (in the cases of $CMV^{+/+}$ transplants) and therefore to be antigen experienced, CD45RO was used as a marker of the memory phenotype.

In addition to characterising the naïve or memory phenotype of T cells, it is possible to segregate T cells into memory or effector memory cells by staining for the CD27 co-stimulatory molecule (respectively $CD27^+$ and $CD27^-$, Hamann *et al*, 1997), which becomes irreversibly downregulated following T cell receptor triggering. Therefore CD45RO in combination with CD27 allows the differentiation of naïve CTLs ($CD45RO^-$ $CD27^+$), memory CTLs ($CD45RO^+$ $CD27^+$), effector memory CTLs ($CD45RO^+$ $CD27^-$) and terminal effector CTLs ($CD45RO^-$ $CD27^-$).

A further and more recently available marker of memory T cell differentiation was described with the chemokine receptor CCR7. CCR7 is a lymph node homing receptor and hence defines a population termed central memory cells, whereas $CCR7^-$ cells (effector or peripheral memory, to distinguish them from $CD27^{+/-}$) express receptors for peripheral circulation and/or migration into inflamed tissues (Sallusto *et al*, 1999).

These three markers, CD45RO, CD27 and CCR7 were used simultaneously with CMV tetramer staining or CD8 specific staining (as a reference for the general cytotoxic population and a guide for the gating of positive and negative cell subsets) to characterise the cytotoxic CMV specific T cell population in SCT recipients.

All patients at risk of CMV reactivation were selected, and staining data representative of a healthy donor and two SCT recipients is shown on Figure III.7.

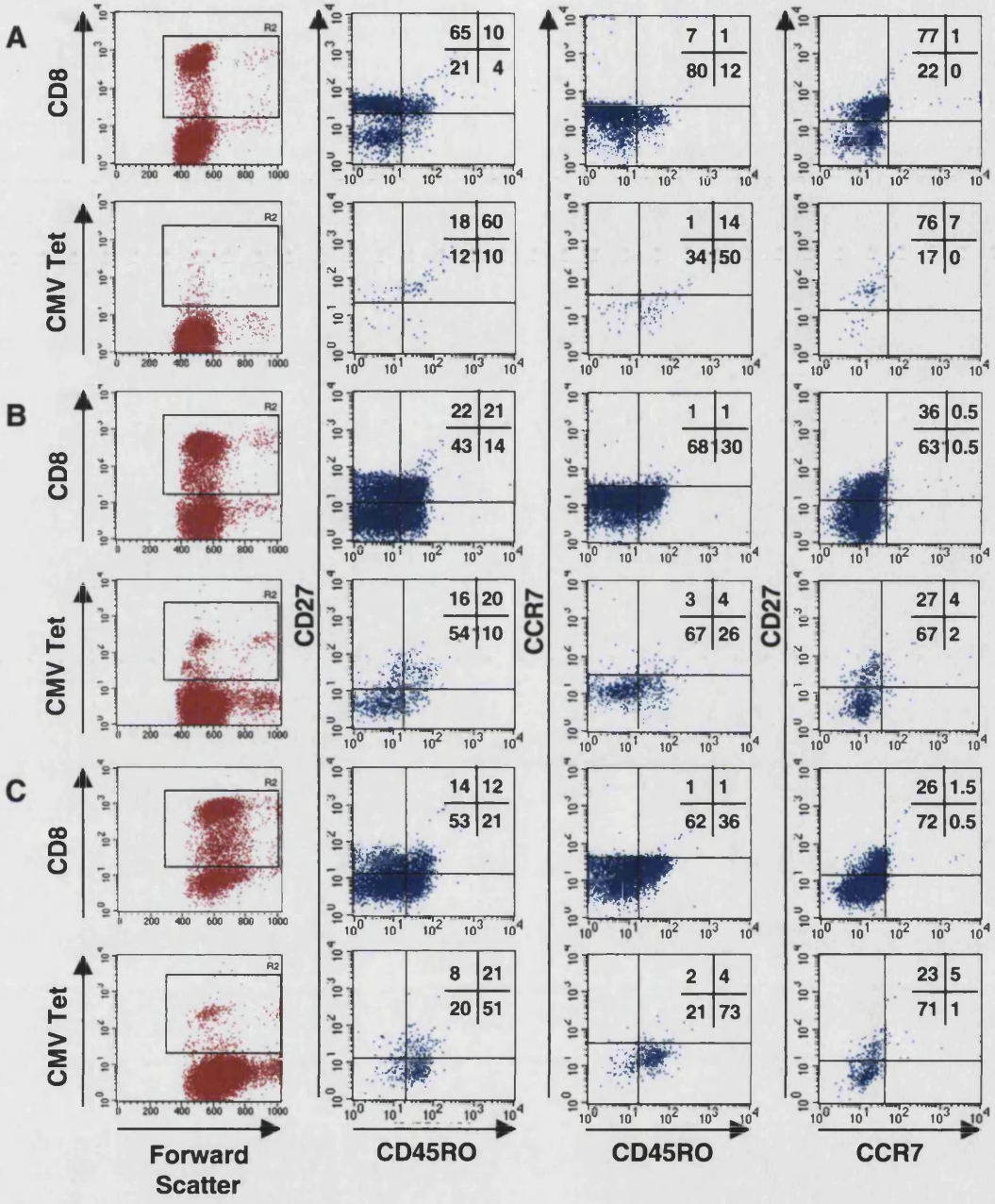


Figure III.7 Phenotypic characteristics of CMV specific Tet$^+$ T cells and CD8$^+$ T cells

Panels are representative of CMV tetramer specific staining and CD8 specific staining (●) A shows staining for a CMV$^+$ HLA-A*0201 healthy donor (HD1), panels B and C show stainings for two CMV$^{+/+}$ SCT recipients, patients 5 and 6 respectively. Positive events were gated and the cells were sub-categorised according to their phenotypic or activation marker expression (●) with antibodies to CD45RO, CD27 and CCR7. The relative percentages of negative and positive events for each sub-category are reported in the top right end corner.

This characterisation was performed retrospectively from frozen PBMC samples, from 8 SCT patients, at timepoints when no current CMV reactivation was detected by PCR screening; the phenotype results for the majority of Tet^+ T cells are summarised in Table III.2.

	Tet^+ cells			$CD8^+$ cells		
	CD27	CD45RO	CCR7	CD27	CD45RO	CCR7
HD1	+	+	-	-	-	-
1	+	+	-	-	-	-
2	+/-	+	-	+/-	+/-	-
4	+/-	+/-	-	-	-	-
5	-	-	-	+/-	-	-
6	-	+	-	-	-	-
7	+	+	-	+/-	+/-	-
19	+	+	-	-	-	-
23	+	+	-	+/-	-	-

Table III.2 Phenotypic characteristics of CMV Tet^+ and $CD8^+$ T cells in 8 SCT recipients

The analysis of the phenotype and activation status of CMV specific Tet^+ T cells and $CD8^+$ T cells was examined in 1 healthy CMV^+ donor and 8 SCT recipients. Tet^+ or $CD8^+$ T cells were gated and the percentage of CD45RO, CD27 and CCR7 positive and negative T cells were determined in each gated population as seen on Figure III.7 the phenotype of the majority of gated cells are reported in the above table.

From the data reported in Table III.2, the following can be drawn. Firstly, in the majority of the patients tested (6/8 high frequencies, 1 heterogeneous, 1 low), CMV Tet^+ T cells expressed high levels of CD45RO, consistent with memory T cells. It is of note that there was a higher proportion of T cells expressing CD45RO in the Tet^+ population than in the total $CD8^+$ population, indicating that the great majority of CMV Tet^+ T cells belong to a memory population.

Secondly, the expression of CD27 seemed slightly less conserved in this patient group with 4/8 SCT recipients with a high proportion of $CD27^+$ cells, 2 with an intermediate proportion and 2 with a low proportion of $CD27^+$ cells. This would suggest that CD27 was heterogeneously expressed on Tet^+ T cells, highlighting a CMV specific memory or effector-memory phenotype CTL population in these SCT recipients. By comparison, the total $CD8^+$ pool had a much more differentiated phenotype with mostly effector type cells that had already encountered a TCR signal.

Finally, a large proportion of Tet^+ T cells did not express CCR7. This was found in all patients, demonstrating that these cells were destined for circulation in the peripheral blood and tissues, as did the majority of the $CD8^+$ pool of cells and also probably memory type cells inherited from the donor.

The results obtained for patient 5 are somewhat unexpected, as the majority of CMV specific T cells appear to consist mainly of terminal effector type cells ($CD27^-$ $CD45RO^-$ $CCR7^-$, while there was no evidence of CMV infection after transplant. Therefore these cells are likely to be of donor origin, but this also raises the question of a possible undetected CMV infection during SCT or early after SCT, or of a possible reactivation in the donor prior to the stem cell harvest.

The predominant phenotype of Tet^+ T cells tested was found to be $CD45RO^+$ $CD27^{+/-}$ $CCR7^-$, consistent with a peripheral memory or effector memory phenotype. This was shown to be true for the majority of the SCT patients analysed. However, it is to be noted that some variability was observed with the CD45RO marker and more so with CD27 marker expression highlighting a degree of heterogeneity in the level of differentiation of Tet^+ T cell population that were observed between different patients.

Detection of BCR/ABL specific T cells in CML patients

The following details the work performed as part of this thesis in the detection of CML specific T cell responses. These experiments were all performed using HLA tetramers. All other data related to the project and the collaborations with other groups will be detailed in the text to ensure clarity in the explanations, and the related publication will be cited as a reference; however, the raw data will not be reproduced here. This project was part of a collaborative study and our contribution at the ANRI consisted in the production of transfected K562 cells for epitope identification and in the production and use of HLA tetramers to investigate the presence of circulating leukaemia specific T cells in these patients.

Elution of KQSSKALQR peptide from the surface of a CML b3a2+ cell line

The work described in this paragraph was performed at the ANRI and in collaboration with our colleagues from Nottingham University prior to this PhD project.

The erythroid lineage cell line K562 does not express HLA molecules at its surface as a consequence of its early differentiation state. It is however positive for the b3a2 BCR/ABL chromosomal translocation found in approximately 40 % of CML patients. This translocation gives rise to transcripts encoding for the p210 b3a2 protein

of BCR/ABL, producing a novel protein in CML patients. The K562 cell line was transfected at the ANRI with a plasmid encoding for the full length HLA-A*0301 molecule. Cell surface expression was confirmed by staining with the HLA-A3 specific monoclonal antibody Gap-A3 and with the HLA class I conformational specific antibody W6/32. The expression of the HLA-A3 antigen on the cells surface allowed the binding and presentation of endogenous peptides at the cell surface specifically in the context of the HLA-A*0301 molecules. As no other HLA molecules are present on the K562 cells, all peptide presentation would be focused through this HLA antigen. Once successfully transfected K562 cells had been obtained and a stable cell line established it was then possible to search for the presentation of the KQSSKALQR peptide spanning the BCR/ABL junction, which was thought to be present on cells bearing the b3a2 translocation and that were also HLA-A3 positive. This transfected K562 system resulted in a great simplification of the data obtained for analysis as a very large pool of peptides are normally presented by up to six different classical HLA class I molecules on the surface of a normal cell. The potential presentation of the KQSSKALQR peptide in the context of HLA-A*0301 was investigated by performing peptide elutions, followed by the analysis of the amino acid sequence of the eluted material by tandem mass spectrometry by our colleagues in Nottingham. As a reference for the peptide profile and to account for the cellular background, a control cell line was loaded with excess KQSSKALQR peptide and the mass spectrometry profile was analysed. Peptide eluate from cultured HLA-A*0301 transfected K562 cell line was subsequently analysed, as well as PBMCs isolated from a CML b3a2$^+$ HLA-A*0301 positive patient and both produced identical peptide profiles on the mass spectrometry read-out. This analysis was published in Blood (Clark *et al*, 2001).

It was therefore concluded that the KQSSKALQR peptide could be presented at the surface of CML b3a2$^+$ cells in the context of HLA-A*0301 and consequently was a potential target by the immune system, and in particular by CD8$^+$ cytotoxic T cells.

Detection and stimulation of KQSSKALQR specific cytotoxic T cells

In order to test whether a KQSSKALQR peptide specific CD8$^+$ cytotoxic response could be detected in HLA-A3$^+$ CML patients, HLA-A*0301/KQSSKALQR tetramers were prepared as described in Chapter II.

Representative staining for six CML patients who were b3a2 and HLA-A3 positive are shown on Figure III.8. For some of the CML b3a2$^+$ HLA-A3$^+$ patients (patients 26 and 27), KQSSKALQR tetramer specific T cells were detected, while in

others, the staining was negative or below the detection level of the assay. The mean frequency detected in CML b3a2$^+$ HLA-A3$^+$ individuals was 0.2% of CD8$^+$ T cells, with a range of 0.01 to 1.03%. As expected, the CML b3a2$^+$ HLA-A3$^-$ patient did not stain with the tetramer, neither did 2 CML$^-$ HLA-A3$^-$ patients (one of these patients was CMV Patient 5, who also had a high frequency of CMV tetramer positive T cells).

For some of the patients selected in this study, expansion of the CML specific T cell populations was attempted by our colleagues in Liverpool to assess if these cells could be used in immunotherapy protocols. The stimulation was performed using KQSSKALQR peptide pulsed autologous PHA blast cells as antigen presenting cells (Clark *et al*, 2001). After one or several rounds of stimulation and subsequent *in vitro* cell culture, two samples were tested by staining with HLA-A*0301/KQSSKALQR tetramer (Figure III.8) and showed a significant expansion of the tetramer positive cells. It was presumed that patient 28 who acquired tetramer positive T cells after stimulation had CML specific T cells present in the freshly stained sample but that were likely below the detection level of the assay.

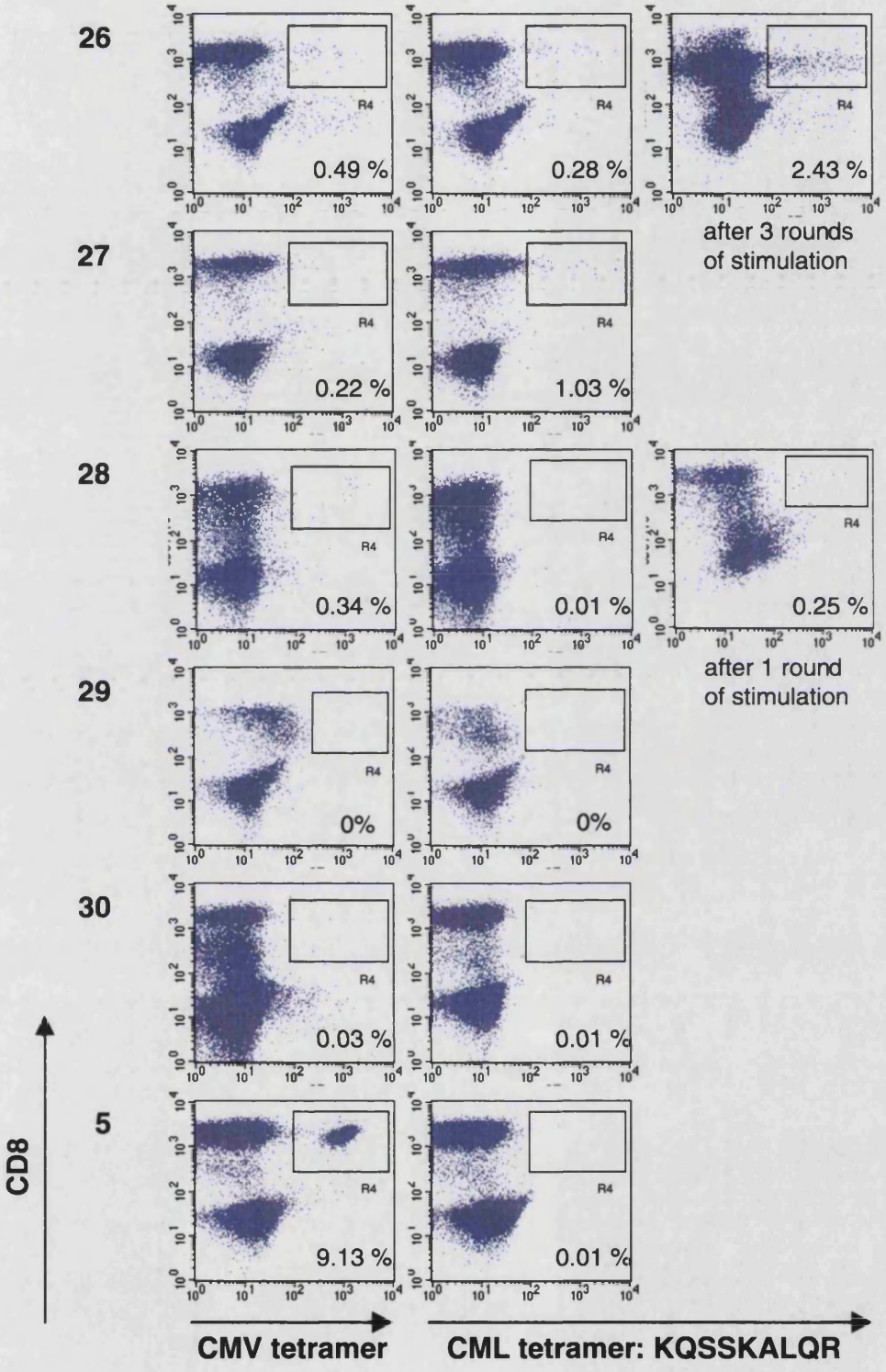


Figure III.8 Detection of BCR/ABL b3a2 junction peptide specific T cells

PBMCs were isolated and stained with HLA-A*0201/AE42 (control reagent and gating reference) and HLA-A*0301/KQSSKALQR tetramers. The data presented is gated on $CD3^+$ events and Tet^+ events are expressed as a percentage of $CD8^+$ T cells. Patients 26,27 and 28 are CML $b3a2^+$ and HLA-A3 positive, Patient 29 was CML $b3a2^+$ but HLA-A3 negative, and Patient 30 was CML^- and $HLA\text{-}A3^-$. Also, CMV Patient 5 was a further control and was CML^-, $HLA\text{-}A3^-$, but was known to have a strong CMV specific T cell response.

This confirmed that CML KQSSKALQR HLA-A*0301 restricted tetramer specific T cells were able to respond to an HLA matched peptide specific stimulus and proliferate *in vitro*.

CML fusion peptide KQSSKALQR specific CD8 T cell cytotoxicity

The cells expanded *in vitro* as described above were also used in chromium release assays to test their specific cytotoxic potential *in vitro*. For three of the patient samples also tested by tetramer staining (patients 26, 27 and 28, Figure III.8), specific killing of lymphoblasts pulsed with peptide, of matched lymphoblasts pulsed with peptide, and most significantly of autologous CML cells was demonstrated. Control autologous and matched lymphoblasts without peptide were not killed in these assays (Clark *et al*, 2001).

This demonstrated that CML peptide specific expanded PBMCs are able to kill peptide pulsed or autologous CMLb3a2$^+$ cells in an HLA restricted manner, highlighting the possibility for the application of a CML tumour specific immunotherapeutic approach.

Discussion

The development and wider availability of HLA/peptide tetramer complexes in the past few years has led to an explosion in the number and diversity of studies focused on antigen specific immune responses. As part of this thesis, HLA-A*0201 and HLA-A*0301 recombinant molecules have been successfully prepared and were used to make HLA/peptide tetramers, which were used to detect antigen specific responses of relevance to haematological malignancies.

Initially, this technology was applied to the detection of CMV specific CD8 T cell responses, and pointed to the fact that this response was directed against only one of the three pp65 peptide epitopes selected both in healthy CMV seropositive individuals and in patients post SCT. While responses can be induced *in vitro* to peptides AE44 and AE45, we saw no evidence of responses *ex vivo*, even though these peptides were found to be better binders of HLA-A*0201 than the AE42 peptide, to which all seropositive individuals tested showed a response. In this respect, the data described in this thesis suggest a significant caveat in relying on the binding of peptides to MHC molecules as the sole basis for epitope prediction. This indicates that the affinity of candidate peptides to the chosen HLA molecule cannot be considered solely from peptide epitope definition, and that knowledge of the antigen or molecule targeted as well as specific

antigen restricted detection and function are indispensable to the definition of an antigenic peptide. Furthermore the study of immune responses in numerous individuals is needed to establish the relative immunodominance of selected antigens in an HLA restricted manner to ensure that the response visualised is representative of the total cytotoxic immune response.

The staining specificity of tetramer reagents was tested and the detection of CMV tetramer specific positive T cells correlated well with peptide specific effector function as measured by IFN-γ ELIspot. However, it was not possible to directly visualise tetramer staining together simultaneously to the peptide specific IFN-γ production as a flow cytometry instrument allowing the detection of 4 fluorescent colours was not available at the time that these experiments were conducted. In addition, specific binding and cytotoxicity of $CD8^+$ CMV Tet^+ T cells towards HLA matched and peptide specific targets could be demonstrated *in vitro*. Furthermore, CMV specific T cells have been shown to produce cytokines following virus specific stimulation, even in a CD4 deficient environment (Appay *et al*, 2000). This fact is of particular interest in the context of SCT, as patients often have deficient or abnormally distributed cellular immune responses. These findings were a good indication that the detection of immunodominant $CD8^+$ CMV specific T cell responses in SCT patients could constitute a significant marker of their virus specific immune recovery. CMV specific T cells could be reliably detected in all SCT patients at risk of reactivation, regardless of their donors' serostatus, setting the conditions for a longitudinal study of CMV specific immune recovery post SCT. Similar CMV specific tetramer detection data with samples obtained from healthy CMV seropositive individuals was reported by Gillespie *et al* (2000), which highlighted what seemed to be a degree of functional heterogeneity in these cells, with a substantial proportion being cytotoxic. However, this data may not be representative of *in vivo* cytotoxicity as it was generated using a T cell clone selected and expanded by *in vitro* stimulation, processes that may modify the properties of these T cells. The correlation between antigen specific tetramer staining with effector function and cytotoxic activity has also been demonstrated for other viruses i.e. HIV (Goulder *et al*, 2000), EBV (Burrows *et al*, 2000) and hepatitis C (Lechner *et al*, 2000). The detection and function of antigen specific T cells was also examined in relation to the phenotypic and activation markers in an effort to further characterise their nature and properties. The expression of the CD45RO, CD27 and CCR7 markers was tested for retrospectively in one healthy CMV seropositive individual sample and in 8 SCT recipient samples. The findings confirmed that the

majority of CMV tetramer positive T cells were of memory phenotype, had a heterogeneous expression of the CD27 activation marker ($CD27^+$ memory and $CD27^-$ effector-memory) and were nearly all committed to the peripheral circulation. These characteristics were found to vary amongst this group of immunosuppressed SCT patients. The heterogeneity of memory CMV specific T cells was also demonstrated in healthy CMV seropositive individuals (Gillespie *et al*, 2000; Vargas *et al*, 2001) and in other groups of immunosuppressed individuals: in renal transplant patients (Gamadia *et al*, 2001) and in HIV patients (Champagne *et al*, 2001). After considering all these results, it becomes difficult to categorise CMV specific T cells in previously infected individuals, although a common feature would be a relatively differentiated memory peripheral phenotype. The case of patient 5 was surprising as the $CD8^+$ CMV Tet^+ T cell population that was detected was mainly $CD27^-$ and CD45 RO^-, which indicates a terminally differentiated cell population, believed to be short lived terminal effectors. This was unexpected, as no CMV replication was ever detected in samples from this patient, therefore there was no evidence of the presence of antigen to induce this type of differentiation. However it is possible to speculate that such a largely expanded differentiated population might have arisen from an expansion from already differentiated donor cells, or perhaps from an undetected episode of CMV infection after SCT. By contrast to findings with CD27 and CD45RO expression, CCR7 expression was found to be consistently negative in $CD8^+$ CMV Tet^+ T cell populations. However, CCR7 expression was shown not to influence immediate effector function or cytotoxicity in the context of recall memory responses both in humans (Sallusto *et al*, 1999) and in mouse models (Unsoeld *et al*, 2002). However, recent studies investigating T cell differentiation pathways have pointed CD27 as being a marker to be investigated further. Tomiyama *et al* (2002) have shown that CMV specific $CD8^+CD45RA^-CD27^-$ T cells were producing more cytokines (IFN-γ, TNF-α) than $CD8^+CD45RA^-CD27^+$ cells for a comparable cytotoxic capability. Additionally, Campbell *et al* (2001) have shown that in CD4 memory T cells, the ratios of $CD27^-CCR7^-$ to $CD27^+CCR7^+$ cells varies from tissue to tissue and may correlate with the number of cells actively engaged in antigen recognition within a given tissue. Therefore further investigation of CD27 expression might clarify further memory differentiation pathways and the assessment of the response to active or recurrent infection. Thus most studies of $CD8^+$ Tet^+ T cells have reported a relatively broad phenotypic distribution amongst CD8 T cell subsets that have lost CCR7 expression highlight the fact that an attempt at categorising these responsive cell populations in persistent viral infections may be inappropriate (Hislop *et*

al, 2001; Appay *et al*, 2002). However, the focus on the phenotype of antigen specific T cells in the context of SCT will probably need further be investigation in order to determine the relative proportions and the level of differentiation of cell populations. This may lead to insights with respect to their functional potential and/or life span.

The HLA/peptide tetramers were also used to determine if a tumour specific BCR/ABL fusion peptide CD8 T cell response could be detected in CML patients. In some cases, these T cells were present at very low frequencies compared to those observed in a virus specific response, and in other cases they were not detected. Yet, some proved to be present at levels below the detection limit of the tetramer staining, and appeared to represent very few cells that could subsequently be expanded *in vitro*. Together with the demonstrations that the KQSSKALQR fusion peptide was presented at the surface of BCR/ABL HLA-A*0301$^+$ cells, that it stimulated PBMCs from CML patients, which subsequently specifically killed BCR/ABL targets in a HLA restricted manner, the positive detection of CML specific CD8$^+$ T cells with HLA/peptide tetramers provided the first evidence for a truly tumour specific immune response. However these low cell numbers make tests constructed to address the question there are so few antigen specific T cells very difficult to perform, however ways of enhancing these to favour a strong response must be investigated. The reasons for finding such a limited T cell response in CML patients are likely to be multi-factorial, and there is emerging evidence that the immune system may be affected with for example defects detected in antigen processing by dendritic cells (Eisendle *et al*, 2003). Furthermore, in the case of patients treated for established tumours, many mechanisms are thought to contribute to tumour immune escape. These include the exhaustion of the immune response, the tolerisation of the immune response or simply escape of the tumour by lack of HLA class I expression or lack of appropriate co-stimulation on the surface of the tumour cells.

Although the present study was restricted in the first instance to HLA-A*0301, other HLA class I BCR/ABL fusion peptide specific epitopes have been described: for example HLA-A24 and HLA-A2 (Nieda *et al*, 1998; Yotnda *et al*, 1998). So tumour specific responses could now be investigated using a similar strategy to that described above and published by Clark *et al* in the context of other HLA alleles. However, the BCR/ABL fusion peptide or the fusion protein are not the only potential targets in CML. The other translocation (b2a2) described in Chapter I as well as the whole sequence of b3a2 might be stimulating tumour associated responses. Also, other tumour associated immune responses directed towards antigens which expression is highly up-

regulated in CML have also been reported. For example, Molldrem *et al* (2000) have shown using HLA tetramers that the detection of proteinase 3 (PR1) tetramer specific T cells correlated with a positive clinical response. Similar findings were also demonstrated in the case of the Wilm's tumour protein (WT1) with WT1 specific cytotoxic T lymphocytes able to kill leukaemic progenitor cells (Gao *et al*, 2000), and WT1 specific peptides described by Bellantuono *et al* (2002), although WT1 specific HLA tetramers haven't been used to date. A similar approach was attempted in the study presented in this chapter, with the search for candidate patients who had received SCT and were successfully treated for rejection by DLI. Unfortunately, no HLA-A*0301$^+$ patients who fitted these selection criteria were found during the course of this project.

One or a combination of peptide epitopes could be selected and assessed in immunotherapy protocols with the aim to stimulate and/or transfer tumour specific cytotoxic T cells in a manner similar to that described by Falkenburg *et al* (1999), with the transfer of leukaemia reactive T cell lines. They could also be used in a peptide vaccination strategy as described by Pinilla-Ibarz *et al* (2000). The former protocol involved a leukaemia reactive line that was raised using HLA haplo-identical donor cells stimulated with irradiated tumour cells, and the latter involved the injection of a combination of several HLA Class I and Class II restricted peptides. The best candidates to benefit from either strategy would be the patients with relatively low tumour burden (Olavarria *et al*, 2001) or the patients responding to treatment with IFN-α or Imatinib Mesylate (Gleevec or STI 571), where the tumour burden is decreased substantially therefore leaving more opportunity for an efficient immunotherapeutic approach. Consequently, a combination approach with the use of chemotherapy and immunotherapy may provide improved treatment options for CML patients. Also the application of an alternate strategy is currently being tested with a peptide vaccination trial with the KQSSKALQR peptide currently under way at the ANRI with a collaborating centre in Liverpool. HLA-A*0301 tetramer will allow us to monitor peptide specific T cell responses in the patients who have entered the vaccination trial. The progress of patients who received SCT and who are treated for disease relapse by donor leucocyte infusion(s) or DLI could also be assessed using CML specific and/or associated tumour antigen specific tetramer reagents. This may be the ideal setting to investigate the graft versus leukaemia effect and study the contribution of different types of stimuli for an effective response, including tumour specific, tumour associated and minor histocompatibility antigen specific responses.

Chapter IV

Cytomegalovirus Specific Immune Reconstitution in Recipients of Allogeneic Stem Cell Transplantation

Introduction

In the immunocompetent host, primary CMV infections and viral reactivations are often clinically silent. In contrast, in the case of immunocompromised individuals, such as those patients who have received immunosuppressive conditioning prior to SCT, reactivation of latent CMV or primary infection can lead to potentially life threatening disease, particularly viral pneumonia (Rubin *et al*, 1989). Furthermore, depletion of T cells from the stem cell graft to control GvHD also removes the potential capacity to control latent or *de novo* infection. (Milburn *et al*, 1989; Hertenstein *et al*, 1995; Couriel *et al*, 1996). This remains the case with the most recent methods of direct or indirect T cell depletion with Campath-1H or CD34 selection respectively (Lowdell *et al*, 1998; Holmberg *et al*, 1999).

Despite the development of potent antiviral drugs, CMV infection is frequent post SCT. Prolonged prophylaxis with antiviral drugs, such as Ganciclovir and Foscarnet, is myelosuppressive and is associated with significant toxicity, morbidity and potential mortality due to invasive fungal infection or late CMV disease (Li *et al*, 1994).

Consequently, the two key parameters directly relating to CMV infection post SCT appear to be the virus reactivation status and the anti-CMV specific immune recovery. The two most sensitive up to date methods were applied to measure these parameters.

Assessment of CMV specific immune reconstitution

On the basis of many studies, the CD8 T cell response has been shown to correlate with recovery from CMV infection of patients after SCT (Quinnan *et al*, 1982, Reusser *et al*,

1997; Small *et al*, 1999). This was also demonstrated with an experimental SCT mouse model of murine CMV infection where the reconstitution of CD8 T cells was shown to be essential for the prevention of multiple organ CMV pathology, and where post transplant T cell depletion lead to lethal disease (Podlech *et al*, 1998). Therefore, it is likely that a CD8 T cell response plays a major role in the control of CMV replication.

However until recently, there was a paucity of studies assessing CMV specific CD8 T cell immune recovery post SCT due to the lack of a rapid and accurate direct *ex vivo* method for quantitating CMV specific CD8 T cells. This problem was resolved by using HLA/peptide fluorescent tetramer complexes, as described in the previous chapter, to detect and quantify CD8 CMV T cells specific for a pp65 immunodominant epitope in the context of the HLA-A*0201 molecule. This constitutes the most sensitive method to date that allows the assessment of antigen specific immune responses.

CMV viral load monitoring

On the basis of many studies using a variety of methods including cell or shell vial culture, pp65 antigenemia assay, or polymerase chain reaction (reviewed by Boeckh and Boivin, 1998), the clinical importance of monitoring SCT patients prospectively for CMV infection has been clearly demonstrated. A comparative study by Einsele *et al* (1995), showed that PCR detection preceded culture detection of CMV and allowed a faster and shorter treatment, leading to a lower incidence of CMV disease, a decreased associated mortality and reduced periods of neutropenia induced by anti-viral treatment. This made a pre-emptive treatment strategy using anti-viral therapy one of the main approaches to the treatment of CMV infection and the prevention of CMV disease post SCT.

A similar strategy was applied routinely for the monitoring of the HLA-A*0201 SCT patient group, as described in Chapter II, to assess CMV reactivation or infection by qualitative PCR (Kidd *et al*, 1993). Although this was a very sensitive method to detect CMV reactivation, no information on longitudinal viral load changes could be detected by this method. A retrospective analysis of the patient samples by QC-PCR and subsequently by real time PCR (Taqman) permitted the accurate assessment of CMV viral load in the blood of SCT patients. Both these techniques were found to give comparable results when they were tested in parallel on identical samples at the RFH department of Virology (Emery *et al*, unpublished observations). High viral loads measured using the QC-PCR technique were associated with subsequent CMV disease in SCT recipients (Gor *et al*, 1998), or with symptomatic CMV infection in another

group of immunosuppressed renal allograft recipients (Hassan-Walker *et al*, 1999). However, the relation between CMV DNA viral load in blood and CMV specific immune responses has not been previously examined.

The aim of this chapter is to examine CMV specific immune reconstitution and CMV viral load as well as their temporal relationship in a cohort of HLA-A*0201 SCT recipients.

Results

CMV specific immune reconstitution in SCT patients

Detection and quantification of CMV tetramer positive T cells in SCT patients

After identifying a population of $CD8^+$ T cells specific for the CMV pp65 AE42 peptide in healthy seropositive individuals and in a selection of SCT patients, the cellular immune response to CMV was monitored in a group of HLA-A*0201 SCT recipients (see Chapter II). Patient 24 was taken out of the analysis because the treatment notes were not accessible at the time the data was consulted; this patient died from disease relapse after 134 days post transplant, consequently the notes were microfilmed and stored in the Haematology department records system. Of the 24 patients remaining in the HLA-A*0201 positive group, 19 were considered "at risk" of developing CMV infection post transplant with a seropositive (CMV^+) patient and/or donor (see Chapter I). Thirteen patients were CMV^+ with a CMV^+ donor, 5 patients were CMV^+ with a CMV^- donor, while only one was CMV^- with a CMV^+ donor. Five were at low risk of developing a primary CMV infection post transplant (with both patient and donor seronegative) and were acting as a control population; none developed a primary CMV infection during the monitoring period.

Isolated PBMCs were stained with the CMV pp65 AE42 HLA-A*0201 tetramer (referred to as CMV tetramer thereafter). As shown in the previous chapter, no significant population of CMV specific $CD8^+$ T cells was identified with the AE44 or AE45 peptide tetramers. All patients at risk of CMV infection post SCT had detectable levels of immunodominant $CD8^+$ CMV tetramer specific T cells (Figure IV.1). The CMV specific T cells were detectable as early as day +21 post transplantation in the case of a non T cell depleted HLA matched sibling donor transplant (Figure IV.1, patient 6). None of the 5 CMV^- patients with CMV^- donors contracted primary CMV infection during the monitoring period, and no CMV specific T cells were detected in any of the samples from these patients that were analysed. A striking feature of these data is that the frequencies and absolute numbers of $CD8^+$ CMV Tet^+ T cells observed in patients reconstituting their immunity were often higher than those observed in healthy control individuals (Figure IV.1, red hatched area, shows 95% confidence interval (CI) of the mean value).

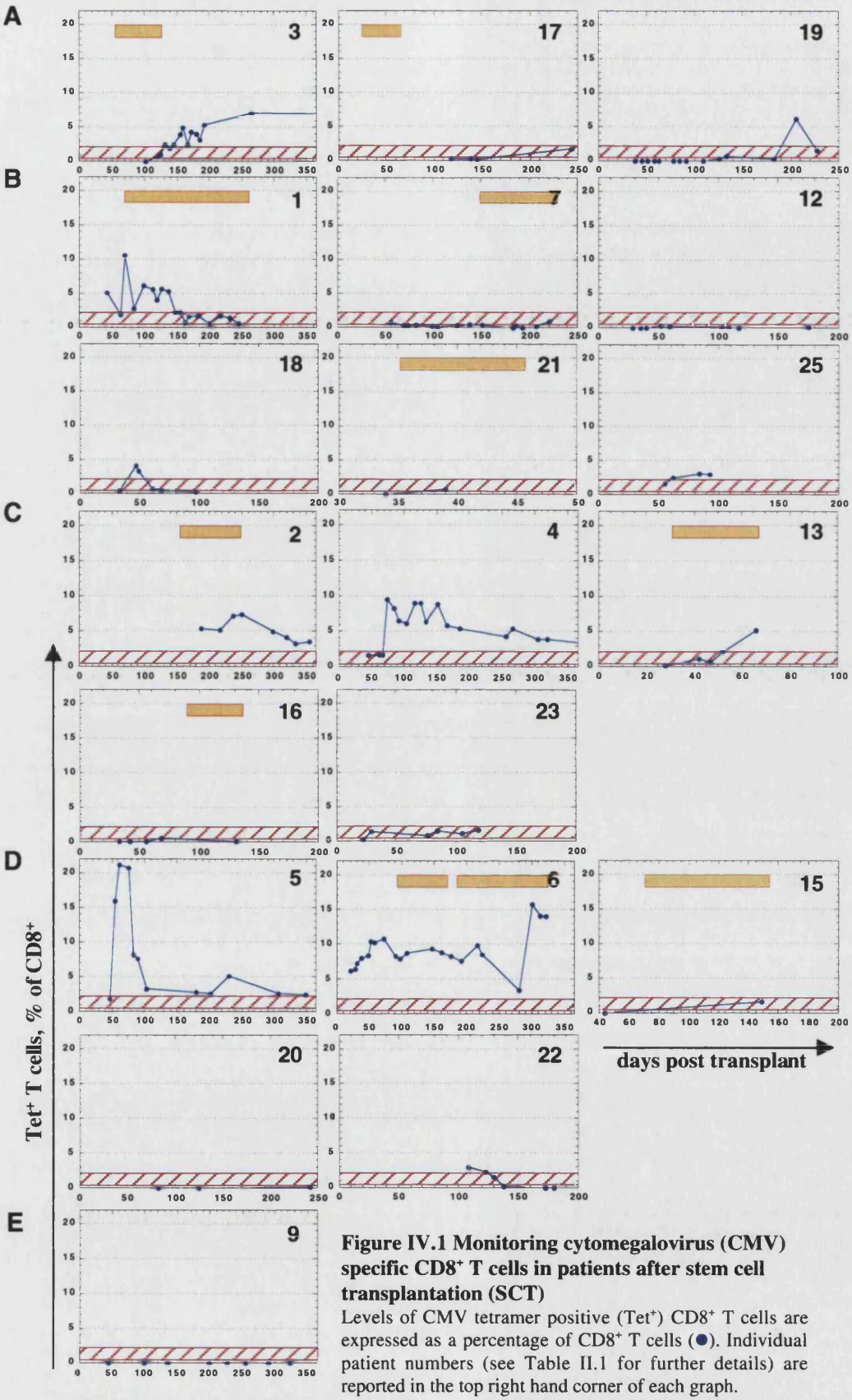


Figure IV.1 Monitoring cytomegalovirus (CMV) specific CD8⁺ T cells in patients after stem cell transplantation (SCT)

Levels of CMV tetramer positive (Tet⁺) CD8⁺ T cells are expressed as a percentage of CD8⁺ T cells (●). Individual patient numbers (see Table II.1 for further details) are reported in the top right hand corner of each graph. Patients were grouped depending on whether they reactivated CMV (A,B or C) or not (D and E) and sub-grouped depending on the level of T cell depletion (TCD) they received: TCD *in vivo* and of graft (A), TCD *in vivo* or of the graft (B) or no TCD (C). For panels D and E, respective levels TCD for each patient were: patients 5 and 15, TCD *in vivo*; patient 6, no TCD; patients 20, 22 and 9, TCD *in vivo* and of graft. Red hatched areas indicate the 95% confidence interval of the mean Tet⁺ frequencies observed amongst healthy individuals. ▭ indicate periods of immunosuppressive treatment with prednisolone. The mean follow up time was 250 days post transplant.

However, of note is the fact that amongst the patients who had CMV infection post SCT, heavily T cell depleted transplanted patients were faced with a much longer period of immune deficiency before their CMV specific immunity was reconstituted (Figure IV.1, Patients 3, 17 and 19, panel A), with no $CD8^+$ CMV Tet^+ T cells detected before 100 days after SCT. This is in contrast with what was observed while monitoring patients who developed CMV infection after SCT and who received either in vivo TCD or received a TCD graft where $CD8^+$ Tet^+ T cells. They were found in the majority of cases to increase rapidly after SCT with the same levels as the frequencies observed in healthy individuals or above (Figure IV.1, panel B). A similar observation can be made for these patients who developed CMV infection after SCT but did not receive any TCD, where higher frequencies of $CD8^+$ Tet^+ T cells can be observed compared to the previous cohort (Figure IV.1, panel C). By contrast to the two groups described above, patients who did not have any detected evidence of CMV infection after SCT appear to be subdivided into two groups (Figure IV.1, panel D). The first group consists of patients 5 and 6 who were shown to have high frequencies of $CD8^+$ Tet^+ T cells from early time-points after SCT and were also long-lasting. These may have arisen from a large expansion of these particular cells in the early reconstitution of the peripheral T cell pool after SCT, or could be due to an undetected CMV challenge. The high levels of $CD8^+$ Tet^+ T cells observed in these two patients might be mediating the immune protection against CMV infection. The second group, which consisted of patients 15, 20 and 22 who did not develop CMV infection after SCT, did not have elevated frequencies of $CD8^+$ Tet^+ T cells (or often below these observed in healthy individuals). This might be representative of the absence of any CMV antigenic challenge in these patients after SCT during the period of monitoring.

These observations led to the comparison of the frequencies of $CD8^+$ Tet^+ T cells observed in healthy individuals with these observed in patients after SCT.

A formal analysis of the difference between the frequencies measured in healthy individuals and the ones measured post SCT was carried out using the Mann-Whitney U test, as the data sets were shown not to have a Gaussian distribution after a Normality test was performed (Figure IV.2). The mean frequency of Tet^+ T cells in SCT recipients was 3.2% (95% CI, 2.71-3.7%; p=0.0956), or 39.10×10^6 Tet^+ cells/l of blood and was found not to be different from the mean frequency observed in healthy individuals: 1.3% (95% CI, 0.54-2.06%) or a calculated value of 8.7×10^6 Tet^+ cells/l of blood (based on an average T cell frequency of 0.67×10^6 T cells/l of blood in healthy individuals).

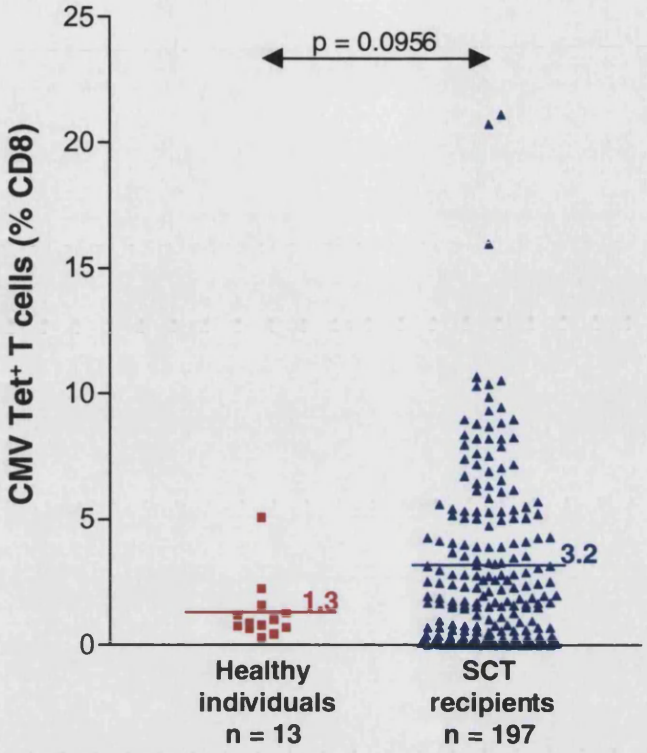


Figure IV.2 Comparison of cytomegalovirus tetramer positive CD8⁺ T cell frequencies in healthy individuals and in SCT recipients

Representation of the CMV Tet⁺ T cell frequencies observed in a control group of healthy individuals (■) and the measurements obtained while monitoring 19 SCT patients at risk of CMV infection post SCT (▲). Bars indicate the mean values. Measurements are frequently higher in SCT recipients than in healthy individuals, although this is not statistically significant (Mann-Whitney U test).

These high frequencies may be attributable to several distinct phenomena post SCT: 1) the re-population of the periphery by haematopoeitic cells, most of which will be mainly driven by peripheral expansion; 2) the expansion of antigen specific T cell pools upon antigenic stimulation; 3) the non-specific proliferation due to bystander activation driven by local inflammation or GvHD; 4) the lack of competition from other antigen specific T cell clones; or even a combination of some or all these possible causes. Therefore the interpretation of these high frequencies is not straight forward and other parameters need to be investigated in parallel with the estimation of the frequency of Tet⁺ CD8 T cells to examine the expansion of specific T cells upon specific antigen stimulation in the context of SCT.

CMV specific CD8 T cell detection and effector function

For 18 out of 19 of the patients at risk of CMV reactivation, PBMC samples were cryopreserved at timepoints when Tetramer positive T cells had been detected by fresh staining. They were subsequently thawed and put back in culture briefly before performing a CMV tetramer staining in parallel with an IFN-γ ELIspot assay. This allowed the assessment of the relation between the presence of CMV specific T cells and peptide specific effector function. The results of this comparison are presented in Figure IV.3. There was good positive correlation between the frequency of tetramer staining and the CMV peptide specific effector function with a Pearson's coefficient r=0.92. This suggests that in SCT patients as in healthy individuals (see Chapter III), the level of CMV Tet^+ $CD8^+$ T cells reflects the capacity of CMV antigen specific T cells to secrete IFN-γ upon specific stimulation in SCT patients.

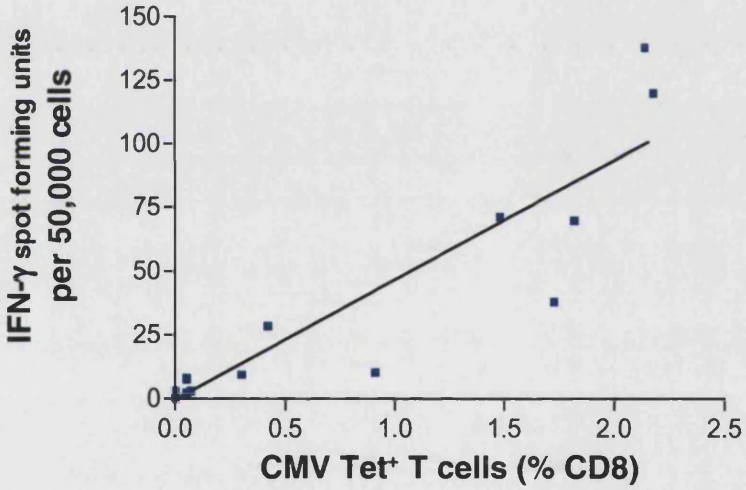


Figure IV.3 Correlation between CMV tetramer $CD8^+$ T cell frequencies and CMV specific effector function

Retrospective analysis of Tet^+ $CD8^+$ T cell frequencies and CMV specific effector function assessed by IFN-γ release mediated by peptide specific stimulation was performed for 18 patients at risk of CMV reactivation post SCT (■). Both groups were tested to confirm their Gaussian distribution before carrying out a Pearson's correlation test. The data sets showed a good level of correlation with a Pearson's coefficient r=0.92 (95% CI, 0.78-0.97; $p<0.0001$) and $r^2=0.84$.

To test whether this finding remained constant throughout the immune recovery period post SCT, three patients were selected for whom regular samples had been cryopreserved. Retrospectively, a time-course analysis of the CMV specific tetramer staining and the peptide specific effector function was then performed. The results are

presented in Figure IV.4. A good correlation between tetramer positive T cell frequency and specific effector function was observed over time. Relatively high frequencies of $CD8^+$ CMV Tet^+ cells and significant IFN-γ production were detected before 100 days post SCT for patients treated with moderate or no T cell depletion with Campath-1H (patients 18 and 23 respectively). This data is consistent with the clinical observation that patients who undergo transplants from HLA matched donors (require only low levels of T cell depletion for GvHD prophylaxis) and recover immune functions relatively early post transplant. However, the recovery of both $CD8^+Tet^+$ positive cells and IFN-γ production were significantly delayed in more heavily T cell depleted individuals (patient19). The frequency of tetramer positive T cells increased after 100 days post transplant, and the IFN-γ specific release remained below 20 spot forming units (sfu) per 50,000 cells tested throughout the period of analysis. This finding is consistent with clinical observations of late immune recovery seen for recipients of grafts from unrelated donors and/or of grafts with some degree of mismatching when more intense conditioning and high levels of T cell depletion will be performed.

Together with the correlation obtained for a single time-point assayed in 18 patients post transplant (Figure IV.3), the data presented highlight the relationship between the frequency of $CD8^+$ CMV Tet^+ T cells and CMV peptide specific effector function, suggesting that both these assays reflect the degree of immunity to CMV.

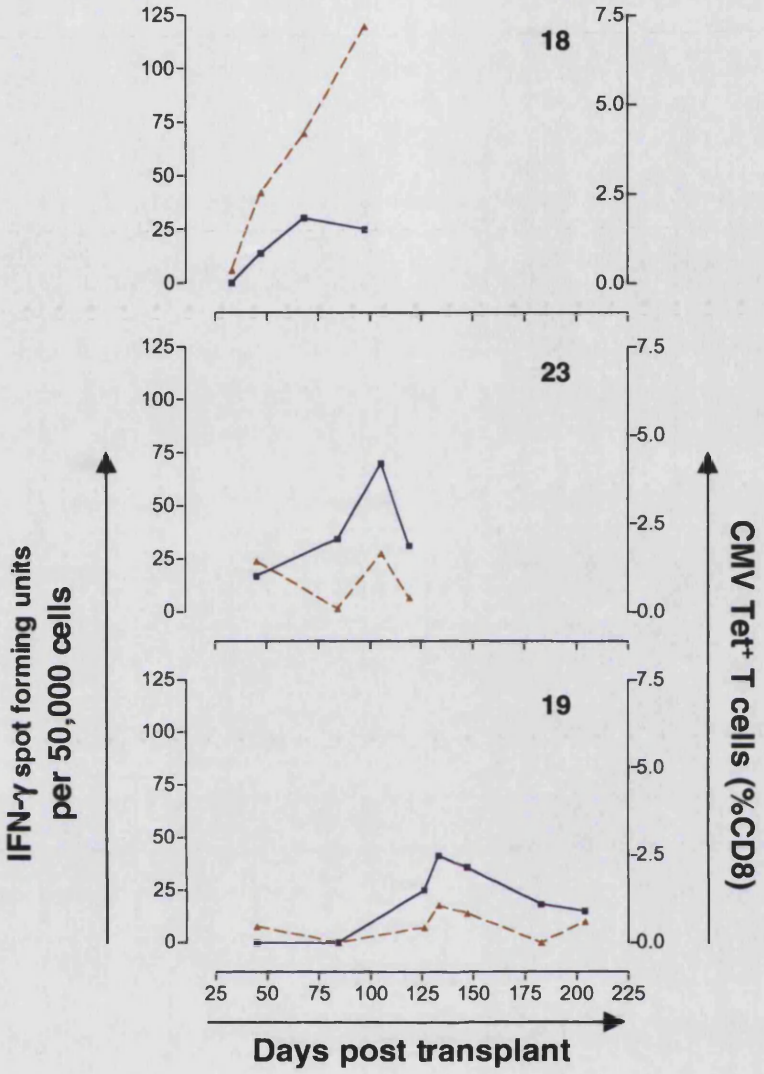


Figure IV.4 Timecourse analysis of CMV tetramer staining and effector function

Simultaneously represented are the Tet^+CD8^+ T cell frequencies (■,—) and the CMV specific effector function as measured by IFN-γ release following overnight peptide specific stimulation (▲, _). Representative patients shown are: patients 18 and 23 after HLA identical sibling SCT, patient 19 after a single major HLA antigen mismatched SCT from an unrelated donor (individual patient numbers are reported in the right hand corner). By contrast to the analysis shown on Fig IV.1 that was performed on freshly isolated PBMCs, the data in this figure was generated by retrospective analysis of cryopreserved samples that were briefly cultured prior to testing. This freeze/thawing process is therefore the cause of the discrepancies that can be noted between Fig IV.1 and Fig IV.4. The data reported on each figure represents the accurate measurement of relative percentages of CMV Tet^+ T cells in each sample tested in separate experiments and therefore cannot be directly compared.

The successful detection of functional $CD8^+$ CMV Tet^+ T cells in SCT recipients led to the examination of the status of CMV infection post SCT in relation to the type of treatment received, particularly to the degree of T cell depletion used. This would enable the study of the relation between these parameters and their potential influence on each other.

Assessment of CMV infection post SCT

The occurrence of CMV infection post transplantation was determined by consultation with the clinical team treating the patients on a regular basis during the sample collection period. Patient records were also accessed to obtain the results of the qualitative PCR performed in the routine department of Virology at the RFH. This allowed the retrospective analysis of the quantitative CMV viral load determined by real time PCR (Taqman, see Chapter II).

As shown previously, of the 5 CMV^- patients receiving a CMV^- graft, none developed primary CMV infection. Amongst the 19 patients "at risk" of CMV infection post SCT, 13 (or 68%) had CMV infection during the period of monitoring. The data summarising the occurrence of CMV infection and the patient information cited in Chapter II were correlated. It was possible to test if this cohort of 24 patients conformed to the well established concept whereby CMV^+ patients and/or patients with CMV^+ donors are considered "at risk" of CMV infection after SCT. This would establish that the patient cohort studied is representative and that similar results may be obtained from studies with the same number, or a larger number of subjects. The data was entered into a contingency table where the conditions were set to test if the patient and/or donor CMV^+ prior to SCT constituted a risk for the occurrence of CMV infection after transplant. This was verified using a two-sided Fischer's exact test and gave a significant positive result: $p=0.0109$. Therefore this confirmed that in this cohort of 24 patients, patient and/or donor CMV seropositivity was a risk factor for the development of CMV infection after SCT, conforming to the clinical consensus and prognosis.

In all 13 cases where CMV infection was detected after SCT, it is probable that these infectious episodes reflect the reactivation of latent virus originating from the recipient and/or the graft, although re-infection can not be discounted totally. The information collected from patients who presented in the clinic with post SCT CMV infection are summarised in Table IV.1. The mean time to the initial detection of CMV DNA in this cohort of 13 patients was day 47 post SCT, ranging from day 7 to day 179 post SCT. On average, the number of infectious episodes per patient was 3. All 4 CMV^+ SCT recipients with CMV^- donors reactivated CMV, while 10/15 (or 66%) of CMV^+ recipients with CMV^+ donors reactivated CMV, indicative of a possible role for the transfer of CMV immunity from the donor to the patient. This could in turn influence the level and/or rate of replication of the virus. The major factors influencing the number of cells transferred to the patient would be the actual frequency of CMV specific T cells present in the blood from the donor, and the level of T cell depletion

applied in the transplant procedure. Unfortunately, it was only possible to assess $CD8^+$ CMV Tet^+ T cells frequencies in two of the donors from this cohort. The recruitment of donors proved difficult and resulted in low consent rate as the only possible times to request their participation in the study was at an already demanding time of donation for graft preparation or for preparation of donor leucocyte infusions in the clinic. However the two donor samples tested had similar $CD8^+$ CMV Tet^+ T cell frequencies to the ones detected in the cohort of healthy CMV^+ individuals mentioned in Chapter III. They were 1.22% (or $11.6x10^6$/l blood) and 0.97% (or $8.6x10^6$/l blood) of $CD8^+$ T cells that were CMV Tet^+ for the donors of recipients 4 and 5 respectively.

individual patient number	patient/donor CMV serotype	first PCR^+ sample post SCT[a]	number of infectious episodes[b]	Campath-1H T cell depletion *in vivo*/in graft
1	+/-	7	7	No / Yes
2	+/+	53	1	None
3	+/+	7	6	Yes / Yes
4	+/+	56	1	None
7	+/+	179	1	No / Yes
12	+/+	13	2	Yes / No
13	+/+	53	1	None
16	+/+	90	2	None
17	+/+	23	3	Yes / Yes
18	+/+	48	1	Yes / No
19	+/-	9	8	Yes / Yes
21	+/-	43	4	No / Yes
25	+/-	49	2	No / Yes

Table IV.1 CMV infection post stem cell transplantation

This table summarises the characteristics of 13/19 "at risk" patients who developed CMV infection post transplant.
[a] indicates the day when CMV DNA was first detected post transplant (with the transplant day being day 0); [b] indicates the number of infectious episodes defined by periods of consecutive CMV DNA PCR^+ results.

The main common factor that we examined in the cohort of 13 patients who had CMV infection post SCT was the level of Campath-1H T cell depletion. This allowed to determine whether this factor had an influence on viral replication or the occurrence of multiple infectious episodes. Because of the low number of individual patients (13) that were studied with respect to the level of viral replication and the frequency of infectious episodes, the accumulated data is an indicator of experiments that would be of interest for larger cohorts of patient. However one of the first characteristics that can be noticed about the 13 patients who had CMV infection after SCT, was that all patients with

CMV^- donors had without exception repetitive occurrence of CMV infection after SCT compared to patients with CMV^+ donors (Table IV.1). This was analysed by grouping the data in two contingency tables and assessing the likelihood of CMV^+ patients with CMV^- donors to develop more than 2 productive CMV infections after SCT. It was found to be a significant risk factor for developing more than 2 episodes of CMV infection after SCT (n=13, two-sided Fischer's exact test: p=0.021). This result might suggest that in the $CMV^{+/-}$ patient subgroup there seems to be a failure of immune protection or of durable immune protection against CMV infection.

Secondly, the level of CMV replication was examined in samples from patients whom had CMV infection post SCT by real time PCR. DNA viral load quantification was performed on 140 samples, 94 of which were PCR+ by routine qualitative PCR assay. The mean viral load measured was 3.61 log10 CMV genomes/ml of blood, with a maximum load of 5.9 log10 CMV genomes/ml of blood. The results obtained in this cohort of SCT patients were comparable to results obtained by quantitative-competitive PCR in a separate cohort of SCT patients studied earlier at the RFH Department of Virology (Gor *et al*, 1998; Emery *et al*, 2000). The CMV DNA viral load was measured in the samples by real time PCR and all positive results were plotted against the level of TCD. The results of that analysis are presented in Figure IV.5. Both data sets were found to have a Gaussian distribution, and there was no significant difference between the mean CMV viral load measured in samples from TCD (3.57; 95% CI: 3.38-3.76; Student's t test: p=0.49) or non-TCD transplants (3.81; 95% CI: 2.83-4.80). There was also no difference between the peak CMV DNA viral load detected in samples from TCD or non-TCD transplants (data not shown). Although this data is only indicative due to the small number of patients studied, it highlights the fact that T cell depletion probably does not contribute significantly to the extent of virus replication during active CMV infection. This is likely due to the frequent viral load monitoring (up to 4 times a week) and prompt treatment with antiviral agents (Ganciclovir and/or Foscarnet). These drugs are very efficient and achieve suppression of viral replication very rapidly. Consequently, the rise in viral load detected during the course of CMV infection is rapidly curtailed by the treatment (see Figure IV.8); however higher viral loads would probably be observed without appropriate treatment or in the rare case of resistant mutant virus. This might be exacerbated in the case of TCD patients and might lead to

higher CMV related mortality, as was the case prior to efficient CMV control (see Chapter I).

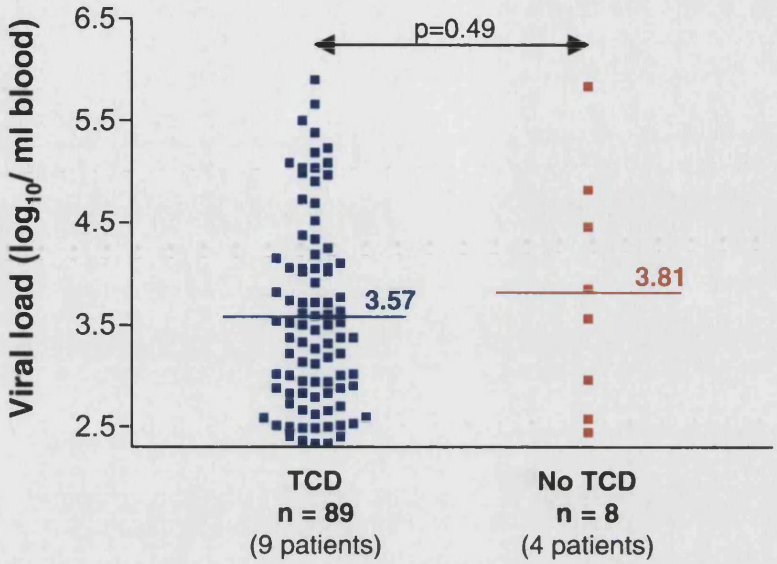


Figure IV.5 CMV DNA viral load measurements in SCT patients with CMV infection

CMV DNA viral load was measured retrospectively for 13 patients who developed CMV infection post SCT. The results were separated depending on T cell depletion (TCD) with Campath-1H (TCD,■; no TCD,■). The 2 data sets had a Gaussian distribution. No correlation was found between the two variables (Student's t test). Bars indicate mean values.

However, it is likely that despite adequate treatment with anti-viral agents, productive CMV infection will reoccur until adequate immune protection has been reached. As immune recovery was found to be slower for patients who received T cell depletion, the actual number of CMV infection episodes could be higher post SCT. This was analysed for the cohort of 13 patients who reactivated CMV. Recipients of T cell depleted transplants tend to have more episodes of CMV infection post SCT than recipients of a non-T cell depleted transplant (Figure IV.6, panel A). The number of non-TCD patients in this group was to small to carry out a Normality test, so a Gaussian distribution was assumed to carry out a Student's t test and compare the mean number of infectious episodes. There is a tendency for these patients to have more infectious episodes even if this does not reach statistical significance (Student's t test: p=0.089).

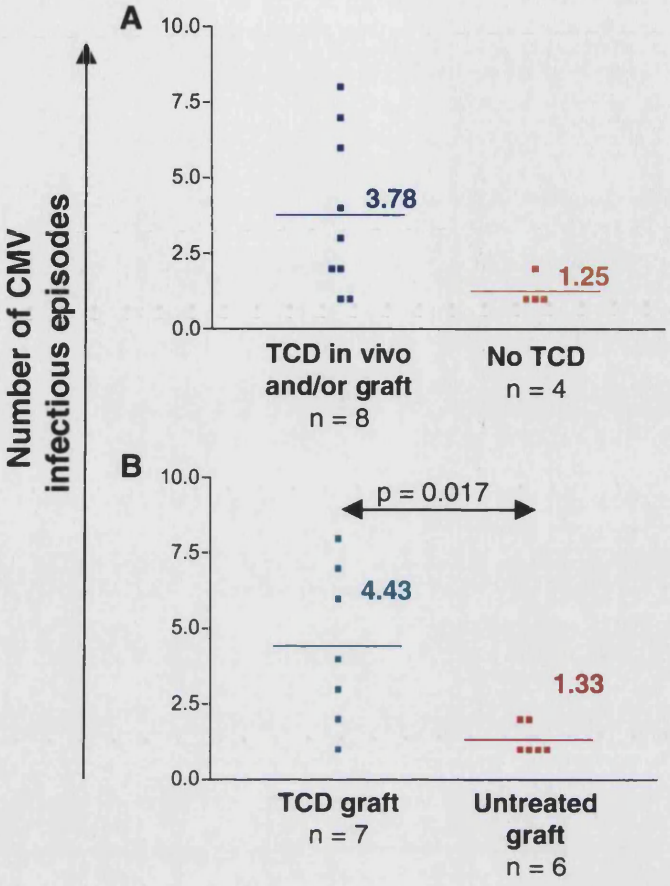


Figure IV.6 Comparison of the number of CMV infection episodes post SCT with the level of T cell depletion

13 patients who had CMV infection post SCT are shown. They were segregated into patients who received T cell depleted transplants with Campath-1H (in vivo and/or graft, ■) or not (■, A), and between patients who received a T cell depleted graft only (■) or no T cell depletion (■, B).

Nevertheless, when T cell depletion of the graft only was considered without taking in vivo T cell depletion into account, it was possible to see a difference between patients who received a T cell depleted graft treated with Campath-1H and patients who received an untreated graft (Student's t test: p=0.017, Figure IV.6, panel B). The mean number of CMV infection episodes was 4.43 episodes (95% CI:1.99-6.86) for T cell depleted graft recipients, whereas it was 1.33 episodes (95% CI: 0.79-1.87) for untreated graft recipients. This was also in accordance with the results of the analysis of the data using contingency tables and assessing whether a TCD graft was a risk factor for developing more than 1 or more than 2 CMV episodes in the 13 patient who had CMV infection after SCT. A similar test was carried out to determine the relative risk of

multiple CMV infection for patients who received *in vivo* TCD. Although the results were not statistically significant, either for a TCD graft (two-sided Fischer's exact test: p=0.1026) or for in vivo TCD (p=0.5649) in the case of more than 1 CMV episode after SCT, they seemed to point to a greater impact of a TCD graft than *in vivo* TCD. This was confirmed when the factor "more than 2 CMV episodes" was tested in a similar way, and a significant risk of having more than 2 CMV episodes after SCT was found for patients who received TCD grafts (n=13; two-sided Fischer's exact test: p=0.0210), but was not a risk factor for in vivo TCD (p=0.2939).

This further indicates that the level of T cell depletion received in the transplant procedure may influence the re-occurrence of infection post transplantation. The differences were detected even in a very small patient population highlighting the fact that these parameters should be investigated in larger patient cohorts.

Both CMV specific T cell immunity and CMV replication were studied in the cohort of 19 patients "at risk" of developing CMV post SCT to assess if immune and viral monitoring could point out the dynamics of these variables and contribute to the development of new treatment strategies.

Relationship between the detection of $CD8^+$ CMV Tet^+ T cells and CMV DNAemia

Initially, the relationship between the detection of CMV Tet^+ $CD8^+$ T cells and CMV DNA in peripheral blood was examined. After the last patient sample was tested, analysis of CMV tetramer and positive CMV DNAemia measured simultaneously on the same day was only available for 24 individual blood samples for 6 of 13 patients who developed CMV infection post SCT, as detected by high level CMV replication (>200 CMV genomes/ml of blood). This was in part due to the treatment strategy that was applied, which consisted of viral monitoring up to four times a week, prompt treatment with anti-viral agents following two consecutive positive results, and an immunological assessment with CMV tetramers that was restricted to once a week. Treatment with immunosuppressive drugs to prevent GvHD could also impact on the number of $CD8^+$ CMV Tet^+ T cells, which made univariate analyses of this data inappropriate.

However, the relationship between the absolute quantity of $CD8^+$ CMV Tet^+ T cells in the presence or absence of CMV DNA in the blood of patients "at risk" of CMV infection post SCT was investigated. Patients were divided into 2 groups for this analysis. The first group consisted of patients who had evidence of CMV infection; the

second consisted of patients who were "at risk" of CMV infection but had no evidence of CMV DNAemia after SCT (Patients 5,6,15,20 and 22). In addition, the number of $CD8^+$ CMV Tet^+ T cells in patients who had CMV infection post SCT was analysed separately during episodes of DNAemia and when no viral DNA could be detected by routine and/or real time PCR. The results of these analyses are shown in

Figure IV.7. The data sets did not have Gaussian distributions, so the difference between sets was assessed using the non-parametric Mann-Whitney U test. The number of $CD8^+$ CMV Tet^+ T cells was the lowest in patients with concurrent CMV DNAemia (mean, $8.8x10^6/l$; 95% CI: 3.36-14.25); whereas in the same group of 13 patients, the number of $CD8^+$ CMV Tet^+ T cells was significantly higher when CMV DNAemia was not detectable (mean, $23.8x10^6/l$; 95% CI: 18.95-28.79; p=0.004). In the cases of patients for whom no CMV infection could be detected on the basis of PCR positivity in blood, the number of $CD8^+$ CMV Tet^+ T cells was $55.5x10^6/l$ (95% CI: 34.62-76.46). This value was significantly higher than the level detected in patients with CMV DNAemia, either during episodes of high CMV loads ($p<0.0001$) or during PCR negative periods (p=0.041).

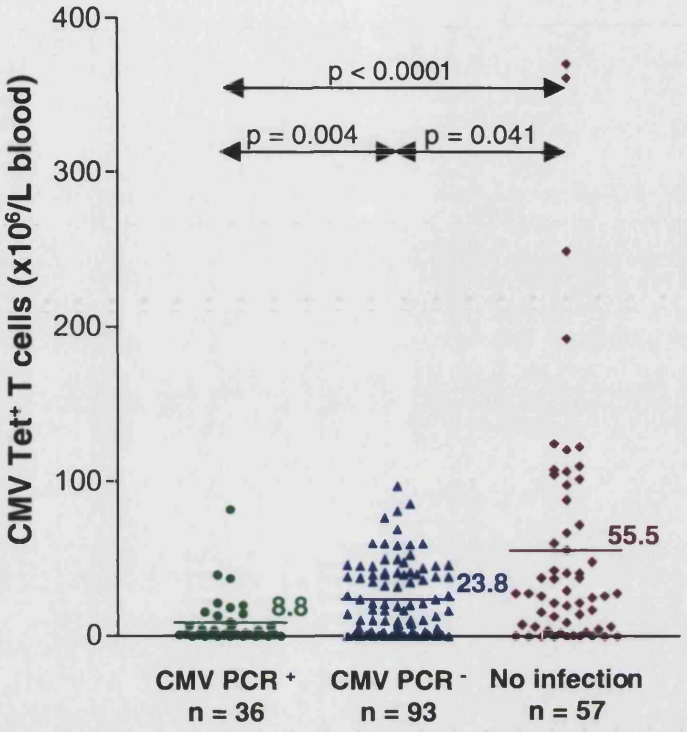


Figure IV.7 Comparison between the number of CD8⁺ CMV Tet⁺ T cells and the CMV infection status in SCT recipients

Levels of CD8⁺ CMV Tet⁺ T cells in SCT recipients who had CMV infection post SCT, at time-points when CMV DNA was detected by PCR assay (●) or when the PCR result was negative (▲), respectively indicating an active or latent phase of infection at the time of the CMV Tet measurement. These were also compared to the Tet measurements obtained while monitoring patients who did not have active CMV infection post SCT (◆). All data sets are statistically different as determined by the Mann-Whitney U test.

These results suggest that the number of CD8⁺ CMV Tet⁺ T cells detected in the blood is representative of the level of immunity to CMV. Although a direct comparison of the number of CD8⁺ CMV Tet⁺ T cells and CMV DNAemia was not possible, a longitudinal analysis of these parameters was carried out for patients with CMV infection post SCT.

Monitoring $CD8^+$ CMV Tet^+ T cells and CMV DNAemia post SCT

Overall, in 15 of 19 patients "at risk" of CMV infection, a similar response pattern was observed with an increase in CMV specific $CD8^+$ T cells after SCT. This correlated in most cases with an increase in CMV load. Three patient cases were chosen to illustrate the dynamics of these two variables post SCT and their relation to each other as well as to the type of transplant and the treatments received.

In the first example, (Figure IV.8, patient 4), the donor had $11.6x10^6$ (or 1.22%) peripheral $CD8^+$ T cells/l of blood staining positive with the CMV Tet. Since the SCT procedure for this patient did not involve any T cell depletion with Campath-1H, a proportion of these cells would have been infused into the patient together with the graft. Nevertheless, CMV still reactivated, with $CD8^+$ CMV Tet^+ T cells present at $5.5x10^6$ T cells/l (1.59% of $CD8^+$ cells) at day +45 and increasing sharply, to reach $15.5x10^6$ T cells/l (1.69% of $CD8^+$ cells) at day +63 post SCT, after CMV infection occurred from days +56 to +67. In subsequent blood samples from this patient, the $CD8^+$ CMV Tet^+ T cells continued to increase, peaking at $86x10^6$ T cells/l at day +86 (8.27% of $CD8^+$ T cells), and then slowly decreased, to stabilise at around $35x10^6$ T cells/l (5% of $CD8^+$ T cells) from day +134. This is representative of CMV^+ patients with CMV^+ sibling donors, with no or mild T cell depletion and for whom CMV infection post SCT stimulates an adequate immune response rapidly restoring viral latency.

In the case of a single HLA-C allele mismatched transplant from an unrelated donor (Figure IV.8, patient 3), a considerable delay in specific $CD8^+$ CMV Tet^+ T cell recovery post transplant was observed. This transplant required more intensive conditioning as well as both *in vitro* and *in vivo* T cell depletion with Campath-1H. Despite multiple periods of CMV reactivation from day 0 to day +96 post transplantation, no $CD8^+$ CMV Tet^+ cells were detected in this patient at day +102 when the patient entered the study. This was thought to be due to a combining effect of the type of transplant, the intensive T cell depletion and also to the steroids administered from day +53 to day +128 for treatment of acute GvHD.

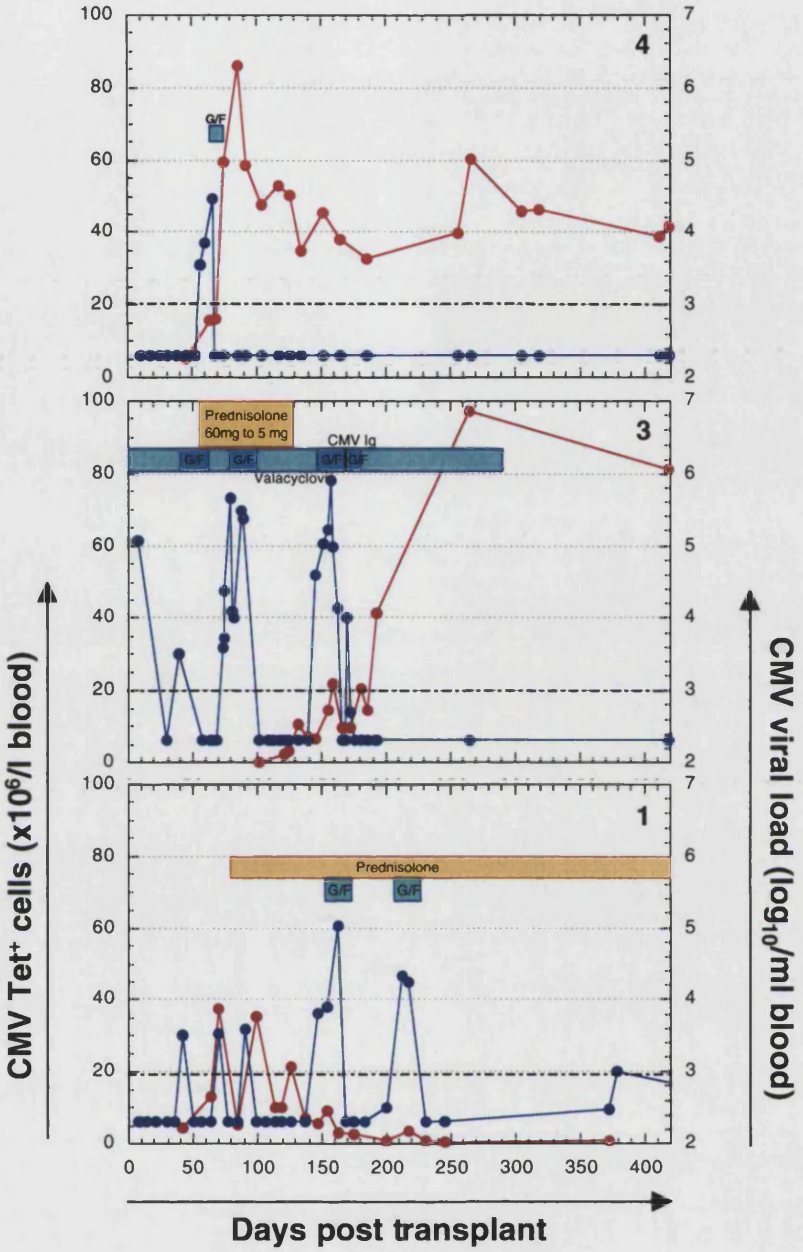


Figure IV.8 Monitoring of CMV specific T cell immunity and CMV viral load

The absolute number of CMV Tet^+ $CD8^+$ T cells ($x10^6$/l of blood, ●) and the quantitation of CMV viral load (log_{10} genomes/ml of blood, ●) are represented as a function of time post SCT. Representative patients are shown (individual patient numbers are reported in the right hand corner of each graph): patient 4 after a HLA identical sibling peripheral blood stem cell transplant; patient 3 after a single allele mismatch (HLA-C) unrelated donor bone marrow transplant; patient 1 after a sex mismatched HLA identical sibling peripheral blood stem cell transplant. Also mentioned on the graphs: CMV Ig, administration of CMV immunoglobulin; ■ G/F: periods of treatment with antiviral agents Ganciclovir and/or Foscarnet; ■ periods of treatment with Valaciclovir; ▭ periods of treatment with prednisolone.

Nevertheless, CMV specific $CD8^+$ T cells numbers recovered from day +145 and were maintained at high levels thereafter (>20 x 10^6 cells/l or 4% of $CD8^+$ T cells) and the patient was protected against further reactivation without need for antiviral help. CMV did not reactivate again after the last reported episode. This illustrates well the significantly delayed recovery of CMV specific T cells after unrelated donor transplants to patients "at risk" of CMV infection post SCT. These cases require extreme clinical vigilance as CMV infection remains a major concern until adequate immune protection is re-established.

In the case of a CMV seropositive patient with a CMV seronegative donor, no antiviral treatment was administered, as the patient never had two consecutive CMV PCR positive results during the early three episodes of reactivation. Each of these increases in viral load was associated with transient elevations of $CD8^+$ Tet^+ T cells, although in each case the levels returned essentially to baseline on clearance of the virus. It is likely that the response measured at that time represented a primary response to CMV mediated by naïve donor $CD8^+$ CMV specific T cells (Figure IV.8, patient 1). Confirmation that the $CD8^+$ Tet^+ T cells were of donor origin was obtained by referring to the results of the *in situ* Y chromosome hybridisation of peripheral blood mononuclear cells. The test was performed to establish the degree of chimerism in this male patient who received a transplant from a female donor and the responding cells were found to be predominantly female. At later time-points after SCT, prolonged treatment with prednisolone led to a fall in CMV specific $CD8^+$ T cells over a course of 3 months. At the end of this period, steroid treatment was maintained and the levels of CMV specific $CD8^+$ T cells were insufficient to control the virus and reactivation (at day + 147 post transplant) was no longer associated with an increase in CMV specific T cells. As subsequent episodes of CMV infection occurred, and as no significant increase in $CD8^+$ Tet^+ T cells was observed, repeated antiviral treatment was needed. This particular example illustrates well the lack of persistent CMV specific T cell responses in CMV^+ patients with CMV^- donors. Although $CD8^+$ CMV Tet^+ T cells appear to respond appropriately to antigenic stimulus, these responses fail to maintain in peripheral blood, with decreasing levels associated with recurrent CMV infection.

Additionally, the evaluation of the number of $CD8^+$ CMV Tet^+ T cells at later times post transplant could allow for the prediction of late CMV infection. Late CMV infection or CMV infection that occurred after 100 days post SCT affected 7 of 19 patients "at risk" (37%) of developing CMV infection post SCT; the observations related to these late infection episodes are summarised in Table IV.2.

individual patient number	patient/donor CMV serotype	known mismatch	chronic GvHD steroid treatment
1	+/-	sex	Yes
3	+/+	1 M HLA[a]	Yes
7	+/+	no	Yes
16	+/+	no	Yes
19	+/-	no	Yes
21	+/-	1 M HLA	Yes
23	+/+	no	Yes

Table IV.2 Late CMV infection post SCT

7 patients presenting with late CMV infection post SCT are mentioned in this table. Also shown: the patient and donor CMV serotype prior to transplant, the occurrence of chronic GvHD as well as any known mismatches that could be the cause of the clinically diagnosed GvHD.
[a] Indicates a single major HLA allele mismatched transplant

The common feature amongst all patients who developed late CMV infection resided in the facts that they all developed chronic GvHD, that this condition was treated with high dose steroid drugs (prednisolone or methylprednisolone) for prolonged periods of time. This factor was considered for all 19 patients "at risk" of CMV infection after SCT to test if long term steroid treatment was a risk factor for the development of late CMV infection. Data was summarised in a contingency table and a two-sided Fischer's exact test gave a highly significant result: p=0.004. Therefore this indicated that in this cohort of patients, long term treatment with steroids was a significant risk factor for the development of late CMV infection. Patient monitoring also highlighted decreasing numbers of both T cell counts and $CD8^+$ CMV Tet^+ T cells numbers. After a period that varied depending on the number of $CD8^+$ CMV Tet^+ T cells previously present and the dose and duration of steroid treatment, this led to CMV infection in all 7 patients without exception. Therefore a longitudinal study of $CD8^+$ CMV Tet^+ T cells could be initiated to predict late CMV infection in patients treated with steroids.

Taken together, these examples illustrate the fact that together with accurate detection of CMV DNAemia, the detection of CMV specific T cell responses using HLA tetramers specific for immunodominant epitopes can provide useful information about CMV specific immune reconstitution. The results of this direct *ex vivo* study

(illustrated with the three cases presented above) confirms the association between rising CMV antigenemia and the subsequent rise in the number of $CD8^+$ CMV Tet^+ T cells and is representative of the immune response to CMV. Based on the patient cases studied, a guideline level was deduced that would constitute a protective level of $CD8^+$ CMV Tet^+ T cells in the blood of patients "at risk" of developing CMV infection post SCT. This was set at $20x10^6$ Tet^+ T cells/l of blood and is represented graphically by an interrupted black line on Figure IV.8. This indicative level was deduced from the study presented above, but a more accurate number could be established with the statistical analysis of a much larger patient cohort.

Discussion

From the study of a group of healthy individuals, the detection of CMV tetramer positive T cells was shown to be consistent with the mediation of an antiviral response, confirming previous findings (Gillespie *et al*, 2000; Wills *et al*, 1999). From our panel of healthy CMV^+ individuals, who are asymptomatic, the absolute number of CMV specific $CD8^+$ T cells can be estimated to be in the range of 1.87 to $10.67x10^6$ cells/l. These values are based on the median frequency of CMV specific CD8+ T cells (0.935%) and the average absolute $CD8^+$ T cell count in healthy individuals (0.2 to $1.14x10^9$ cells/l). This seems to account for a larger proportion of the cytotoxic T cell pool being restricted to a single CMV epitope than might otherwise be expected, but this data has also been confirmed by other groups (Gillespie *et al*, 2000). This observation also appears to be in accordance with immune responses directed against other persistent viruses from the herpesviridae family, such as Epstein-Barr virus (Callan *et al*, 1998). The study of 25 SCT recipients presented in this chapter highlighted similar if not higher frequencies and absolute numbers of CMV specific T cells. A number of other studies also showed high frequencies of $CD8^+$ CMV Tet^+ T cells in recipients of SCT (Gratama *et al*, 2001; Cwynarski *et al*, 2001) but also in the context of multiple viral infections in non transplanted HIV^+ patients (Jin *et al*, 2000). This finding in SCT recipients may reflect the requirement for more CMV specific T cells to control CMV infection after SCT, but could also be due to an inflation of the representation of CMV specific T cell clones. These cells might be amongst the first stimulated in the case of CMV reactivation at a time when the peripheral pool starts to reconstitute after SCT, which could be at the origin of these high frequencies. Similarly to the findings for healthy CMV^+ donors as described in Chapter III, a defined correlation between the detection of $CD8^+$ Tet^+ T cells and CMV peptide specific effector function was established in SCT patients "at risk" of CMV infection. Despite the fact that the measurement of IFN-γ release requires *in vitro* stimulation and only detects a fraction of HLA peptide restricted T cells, it can be utilised to demonstrate the peptide specificity of responses by ELIspot or intracellular staining/flow cytometry as shown by Hebart *et al* (2002). Simultaneous tetramer and IFN-γ release can now be performed on a single cell basis by flow cytometry, this should lead to a better understanding of the antigen specific T cell population dynamics and activation requirements with respect to a specific peptide stimulus.

CMV specific $CD8^+$ T cells can be detected at relatively early times post SCT and are associated with the control of viral replication when levels of $CD8^+$ Tet^+ T cells of at least $20x10^6/l$ are reached and maintained. Furthermore the onset of viral replication was found to be associated with low CMV specific $CD8^+$ T cell counts. This implies that the number of CMV Tet^+ T cells may be used to predict episodes of viral replication when they are lower than $20x10^6/l$ or lower. The impact of the level of T cell depletion on CMV reactivation was examined in the patients at risk. A difference in relation to the frequency of occurrence of CMV infection was found when the recipients were T cell depleted with Campath-1H *in vivo* and/or received an *ex vivo* T cell depleted graft. Graft TCD was found to have more impact on the occurrence of CMV infection, especially as it was found to be a significant risk factor for developing multiple reactivation episodes. No impact on the CMV viral load or the peak viral load was detected by comparing each group for the 25 SCT patients studied in this chapter. However the number of patient studied was small and this point should be examined with the study of a larger group of patients. These findings have been confirmed by Suparno *et al* (2002) and Chakrabarti *et al* (2002), who demonstrated a high incidence of CMV reactivation after SCT with an unrelated donor transplant as being a significant risk factor for CMV recurrence and highlighted the potential role of Campath-1H in delaying CMV specific immune reconstitution. Additionally, a study by Qamruddin *et al* (2001) also pointed to the role of Campath-1H when used in the conditioning regimen of transplant procedures and the link to subsequent CMV reactivation post SCT. The study presented in this chapter also pointed to the fact that long term treatment with steroids depletes $CD8^+$ CMV Tet^+ T cells and constitutes a very significant risk factor for the development of late CMV disease.

The majority of active CMV infections observed in patients after SCT are due to reactivation of a latent infection (Pillay *et al*, 1992). It has been suggested that donor marrow from seropositive donors may provide cellular immunity to the recipient thus accounting for the lower incidence of CMV disease in these patients (Grob *et al*, 1987; Gor *et al*, 1998). Data from our study provides further evidence in the support of these observations: with CMV specific responses observed early after SCT in all seropositive patients before *de novo* lymphopoiesis could have occurred. To date, this study provides the first and only formal evidence of an inverse correlation between active CMV infection and the number of $CD8^+$ CMV Tet^+ T cells in patients post SCT. Further, this data demonstrates the benefits of using CMV tetramers to monitor the absolute number of CMV specific T cells in recipients of SCT. Regular CMV tetramer staining allowed

the assessment of CMV immune reconstitution in patients after SCT with levels of $20x10^6$ CMV specific cells per litre of blood (whether the patients have previously become positive or not by PCR) being a good indicator of CMV immunity that could be utilised functionally in a clinical setting. The absolute number of CMV specific T cells was chosen as an indicator of specific immunity rather than the percentage values because the latter can be misleading in the context of SCT as lymphocyte numbers tend to be low and tend to fluctuate substantially, especially in the first six months after transplant. Similarly to the findings for the cohort of 25 SCT patients presented above, a separate study by Cwynarski *et al* (2001) monitoring HLA-A2 and HLA-B7 restricted immunodominant pp65 specific T cell responses as well as pp65 antigenemia associated $10x10^6$ $CD8^+$ CMV Tet^+ cells/l with protection from CMV disease. The protective levels deduced from both this study and the study described in this chapter are of the same order, which supports the concept of the ability to predict CMV infection. The difference between the two figures can be explained in part by the facts that tetramer analyses were performed on freshly isolated PBMC samples in the present study versus cryopreserved PBMC samples in the study by Cwynarski.

Previous studies have shown that the greatest risk of CMV disease is associated with transplantation of a CMV seronegative marrow into a seropositive patient (Grob *et al*, 1987; Winston *et al*, 1985). Furthermore a recent study (Nichols *et al*, 2002) showed that SCT CMV^+ recipients with CMV^+ or CMV^- donors remain at a higher mortality risk than SCT CMV^- patients with CMV^+ donors despite the improvements in antiviral treatment. This study also pointed to a trend to an increase incidence of CMV disease for CMV^+ patients with CMV^- donors, compared to CMV^+ patients with CMV^+ donors, and pointed to a higher risk of death due to bacterial or fungal infection in the former group of patients. The findings presented in this chapter provide further evidence that this might be the case as we have observed a transient increase in CMV/AE42 specific cells seen in CMV^+ recipients who received a transplant from a seronegative donor. This contrasts with the response from patients with CMV^+ donors, where the levels of CMV/AE42 specific T cells remained elevated following virus clearance. These results imply that patients with CMV seronegative donors, although they are able to respond to increases in viral levels, do not seem able to sustain these responses, which would therefore make them more susceptible to recurrent infection. However, we need to study more cases to confirm this point, as the complicating factor in interpreting the failure to maintain elevated levels of CMV specific T cells is that patient 1 developed chronic GvHD, requiring immunosuppressive treatment with prednisolone. Monitoring patients

"at risk" of CMV infection post SCT also established that steroid treatment can deprive patients of effective anti-CMV responses and this may be associated with both recurrent and/or late CMV infection. This fact was also highlighted by a study showing that steroid treatment was the primary risk for rising pp65 antigenemia in SCT patients (Nichols *et al*, 2001). In the cases of patients with CMV^- donors and/or on long term steroid treatment especially, tetramer monitoring has proven to be an important parameter for clinical follow up.

It has been established that the development of GvHD after SCT is associated with CMV infection (Wu *et al*, 1975; Grundy *et al*, 1985), and a synergy between GvHD and CMV infection resulting in catastrophic lung pathology (interstitial pneumonitis, IP) has been postulated. Two mechanisms of pathogenesis in the lung have been proposed: 1) due to uncontrolled viral replication in the lung caused by the absence of CMV specific immune response (Reusser *et al*, 1991; Greenberg *et al*, 1991); 2) due to an immunopathological reaction whereby the immune system might lead to pathology triggered by CMV infection (Grundy *et al* al, 1987; Grundy *et al*, 1988). With the use of potent antiviral agents in prophylactic or pre-emptive treatment strategies, the occurrence of CMV-IP has been reduced very significantly. None of the 25 SCT recipients studied in this chapter developed CMV-IP. However the debate over the relation between GvHD and CMV (i.e. whether GvHD causes CMV infection or vice versa and their relation to the development of CMV-IP) remains. Evidence from this study supports the first hypothesis, as it was shown that high frequencies of CMV specific T cells correlated with recovery from CMV infection and protection from further episodes without any evidence of GvHD or IP. The use of pre-emptive Ganciclovir and/or Foscarnet treatment did not appear to impair immune recovery, despite the fact that Ganciclovir was shown not to affect the down-regulation of HLA class I expression on cells infected by CMV (Grundy *et al*, 1998). Additionally, all the patients who developed chronic GvHD and were treated with steroid immunosuppressive drugs contracted late CMV infection, which would also suggest that the absence of a CMV specific cytotoxic immune response (or its decline) is responsible for CMV reactivation. However, supporting the second hypothesis, there is also firm evidence that CMV has many pro-inflammatory properties including the induction of cytokines (IFN-β, IL6, TGF-β), chemokines (IL8) and adhesion molecules (ICAM-1, LFA-3) that could all contribute to triggering or aggravating GvHD (for a review, see Grundy *et al*, 1998); therefore CMV could be the source of the GvHD reaction in the lung and of subsequent immunopathology resulting in IP. As there was

no evidence of CMV IP in the present study, it is only possible to speculate that in the case of uncontrolled CMV infection in the lung, local viral cytopathogenicity and inflammation may contribute to enhance the number of functional CMV and GvH specific T cells that might both contribute to tissue destruction. However, as CMV IP has become less frequent due to efficient CMV treatment, this hypothesis might not be tested. Despite the significant progress in CMV treatment after SCT, the overall survival of SCT recipients "at risk" of developing CMV infection has not improved significantly. Therefore the need for improved antiviral agents with less toxicity and side effects and/or alternative cellular therapies such as CMV specific T cell transfer remain important areas that warrant further research.

Thus, as seen in this chapter, systematic tetramer monitoring of patients post transplant and correlation with the viral load will allow the design of more efficient strategies for the prevention of CMV infection and/or reactivation. The establishment of a sufficient level of CMV specific $CD8^+$ T cells following SCT will avoid the need for regular screening, unless a sudden drop in total lymphocyte numbers is observed (as is often the case following the initiation of steroid therapy for the treatment of GvHD). This will also help to define a balance between the early use of antiviral drugs, which might prevent disease but also impair bone marrow causing neutropenia, and possibly induce resistance and late disease in the case of prolonged treatment (Li *et al*, 1994; Goodrich *et al*, 1991; Knox *et al*, 1994; Emery *et al*, 2000; Emery *et al*, 2000).

Furthermore, it is now possible to isolate specific T cells from the seropositive stem cell donor for adoptive transfer into the patient at the time of transplant or soon thereafter. Leukocyte transfusion and adoptive transfer of CMV immunity by infusion of specific $CD8^+$ T cells has been used with success (Walter *et al*, 1995; Riddel *et al*, 1997; Witt *et al*, 1998; Einsele *et al*, 2002). The use of the tetramers to specifically isolate CMV effector cells directly from the donor's PBMCs whilst avoiding unwanted $CD8^+$ T cells with other antigenic specificity is clearly feasible and highlights this as a fast and efficient means of obtaining effector cells with reduced handling and minimal culture periods. In addition this may prevent the onset of GvHD which often occurs following the transfer of unselected lymphocytes populations (Papadopoulos *et al*, 1994). Therefore, adoptive transfer should be considered for use in the case of unrelated and haplo-identical transplants as these patients display delayed engraftment and immune reconstitution, and in all patients after cessation of steroid therapy, during which time they require active protection against CMV. Finally, CMV/AE42 tetramer monitoring may also be considered valuable in the investigation of poor graft survival

and post transplant complications in the solid organ transplant setting. In these cases also, adoptive transfer of CMV specific $CD8^+$ T cells will find clinically relevant applications.

Chapter V
Modulation of antigen specific immune responses

Introduction

The successful detection and quantification of antigen specific $CD8^+$ T cells using HLA/peptide tetramer complexes was described in the previous chapters. The specific and stable interaction between HLA/peptide tetramers and their specific TCR can be further exploited to modulate antigen specific immune responses by transferring purified and/or enriched T cells from an immune donor (adoptive transfer), or by directly influencing the antigen specific immune response. This can be achieved by using positive tetramer staining as a selection marker to purify $CD8^+$ Tet^+ cells. Purification methods need to be tested for the establishment of standardised protocols in order to achieve safe T cell adoptive transfer in the context of SCT. Additionally, the specific interaction between HLA/peptide complexes and the TCR in specific immune recognition and activation processes can be exploited to mimic the role of antigen presenting cells with HLA/peptide tetramers. Presenting PBMCs with soluble HLA/peptide tetramers could induce antigen specific TCR oligomerisation and therefore could potentially induce stimulation and/or proliferation if this signal proved sufficient. The possibility of applying HLA/peptide tetramers to the stimulation of antigen specific T cell responses was examined *in vitro*.

HLA tetramers and adoptive transfer

The fact that antigen specific $CD8^+$ T cells bind tetramer reagents leads to the possibility that tetramer binding may initiate the formation of TCR oligomers and lead to signal transduction through the TCR followed by stimulation of the T cell response (Reich *et al*, 1997; Boniface *et al*, 1998). This might constitute a barrier to the clinical application of tetramer purified antigen specific T cells, as unwanted activation induced cell death might be triggered, which would diminish the efficacy of the T cells.

Additionally, some of the tetramer molecules would be present at the cell surface and/or internalised by the T cell. Consequently the components of the HLA/peptide tetramer may still be present at the cell surface and may constitute an immunogenic target themselves, therefore jeopardising repeat treatment. This is likely to be a significant concern as streptavidin (or avidin) is a well described immunogen that induces an antibody mediated immune response. Furthermore, the impact of the *in vivo* transfer of tetramer sorted cells has not yet been assessed in human patients. Consequently, a system using a biotin analogue, 2-iminobiotin, was tested to make tetramer complexes because of its lower affinity for streptavidin compared to biotin. This is aimed at generating tetramer complexes that could be dissociated from Streptavidin after the purification had occurred, using a simple biotin competition wash, therefore releasing HLA/peptide monomers and disrupting HLA/peptide-TCR oligomerisation by decreasing the interaction. This could prevent T cell activation by the tetramer reagent used in the staining and purification of the antigen specific T cell population of interest, as well as allowing for the tetramer reagent to be washed away from the T cells.

In vitro modulation of antigen specific immune responses

Most antigen specific responses that have been described so far are generally characterised by low number of specific cells in peripheral blood. They can even in some instances be below the detection limit of the most sensitive techniques available, especially in cases of tumour specific immune responses (see Chapter III). Therefore whether the targeted T cell population involves low numbers of T cells or the generation of a primary immune response, adoptive transfer of T cells is preceded by a period of in vitro stimulation and expansion. This can be achieved by single cell cloning and antigen non-specific stimulation by cross-linking of the CD3 and CD28 molecules on the cell surface (Dunbar *et al*, 1999), or by using natural antigen presenting cells, dendritic cells, loaded with the specific antigen (Oelke *et al*, 2000; Szmania *et al*, 2001). This is followed by a period of expansion in culture. Both methods have been successfully applied to the generation of virus or tumour specific T cell responses, and dendritic cell stimulated CMV specific T cells were used for *in vivo* adoptive transfer in a study of SCT recipients "at risk" of CMV reactivation (Peggs *et al*, 2002).

However, both methods have significant downfalls. The first strategy targeting CD3 and CD28 for the stimulation of the T cells is not antigen specific, and therefore requires prior single cell sorting. This can be a limitation as the efficiency of flow cytometry sorting is good but not absolute (two cells could end in the one well or could

be discarded if they pass the detector simultaneously), and few centres possess this facility. The second strategy involving the generation of dendritic cells is both labour intensive and time consuming and requires the availability of bone marrow or more commonly of a large volume of blood. Additionally, the number and quality of dendritic cells generated varies considerably on an individual sample basis, and it is not possible to control for any other antigen that might be presented by these dendritic cells despite loading them with an excess of the specific antigen. Other molecules can also be considered for the targeted modulation of antigen specific T cell responses. For example, the induction and maintenance of antigen specific T cell responses could be achieved with TCR signalling with simultaneous cross-linking of co-stimulatory molecules. These can be CD28 as mentioned above, CD40 ligand (or CD40L; Lefrancois *et al*, 2000), which is thought to contribute to the stimulation of primary T cell responses or 4-1BB (Shuford *et al*, 1997; Takahashi *et al*, 1999), which is a novel cell surface molecule thought to be involved in T cell survival mechanisms. Additionally, antigen specific T cells could be targeted for deletion through CD95 to suppress unwanted T cell responses.

In recent years, strategies have been devised in an attempt to replace natural antigen presenting cells with so-called artificial antigen presenting cells that can be used in vitro to stimulate antigen specific T cell responses. For example, mouse fibroblasts can be retrovirally transduced with a single HLA/peptide complex, along with the human accessory molecules CD80 (B7.1), ICAM-1 and LFA-3. These artificial antigen presenting cells elicited strong stimulation and expansion of HLA restricted CTLs, specific for viral antigens or tumour associated antigens (Latouche and Sadelain, 2000). This method appeared to be very reliable for the generation of memory and primary CTL responses, but the main drawback would be that a new artificial antigen presenting cell line would have to be produced for each HLA/peptide combination wanted. Furthermore, the use of a mouse derived cell line might restrict potential applications. A more versatile cell based strategy was described with the use of K562 cells, which belong to the erythroid lineage, therefore do not express HLA molecules at their cell surface, but do express ICAM-1 and LFA-3. These were transfected with the human CD32 Fcγ receptor to allow binding of anti-CD3 and anti-CD28 antibodies to provide a TCR signal and with 4-1BB ligand (Maus *et al*, 2002). These artificial antigen presenting cells were shown to activate and rapidly expand polyclonal or tetramer sorted populations. However the use of a tumour cell line "vehicle" may restrict their

use. Consequently, the possibility of exploiting the specific avidity and properties of HLA/peptide multimers to target antigen specific T cells with the addition of co-stimulation for the activation and proliferation of these cells was examined. This would constitute a simpler and more versatile non-cellular "vehicle" compared to these described above.

In an attempt to create a system that would allow efficient antigen specific stimulation with the possibility of simultaneous co-stimulation, HLA/peptide-antibody chimeramer complexes were tested as a form of "minimalist" artificial antigen presenting system. These complexes basically consisted of a modified HLA/peptide tetramer composed of three biotinylated HLA/peptide monomers and one biotinylated antibody molecule bound to streptavidin. The synthesis and testing of these complexes was described in Chapter II. The ratio of 3 HLA/peptide complexes to 1 antibody molecule was chosen so that specific binding to the TCR would be retained and would be more avid than that of the antibody molecule component, therefore specifically targeting antigen specific T cells for stimulation and co-stimulation. Similar complexes were used previously in a different context aiming at redirecting viral specific T cell responses to tumours that were characterised by the presence of a specific antigenic marker that was targeted by the antibody (Ogg *et al*, 2000; Robert *et al*, 2000). This required constructing virus specific HLA/peptide-antibody complexes (chosen because of a strong antiviral CTL response) in a similar way to that described previously and in Chapter II, but to use an antibody that would specifically target the tumour cell, therefore coating it temporarily with HLA/peptide complexes and mimicking a virus infected cell. Consequently virus specific CTLs would be redirected to destroy marked tumour cells. Antigen specific TCR engagement and virus specific cytotoxicity were maintained in these studies, using tumour cell lines as targets, which represented an alternative approach to tumour immunotherapy. However this system was not described in a more physiological context where both antigen specific T cells and tumour cells were present or in an *in vivo* model, therefore it is not known if this strategy would work *in vivo*.

Successful modulation of *in vivo* immune specific T cell responses has been achieved in mouse models (Maile *et al*, 2001) using transfer of soluble HLA/peptide tetramers. The confirmation that antigen specific immune modulation can be achieved in vitro using HLA/peptide tetramer or chimeramer reagents would provide the first evidence that they might be used for *in vivo* applications as part of the purification

and/or vaccination protocols. Consequently, the possible use of HLA/peptide chimeramers to stimulate antigen specific T cell responses was examined *in vitro* in the present study.

The aim of this chapter is to investigate the possible uses for HLA/peptide tetramer and chimeramer complexes in the modulation of antigen specific T cell responses for the adoptive transfer of selected cells or the direct application of these complexes.

Results

HLA/peptide tetramers and adoptive transfer

Purification of $CD8^+$ Tet^+ T cells

The enrichment of $CD8^+$ Tet^+ T cells can be achieved by selecting tetramer positive cells by flow cytometry or magnetic bead sorting (Keenan *et al*, 2001; Szmania *et al*, 2001). The highest purity (>90%) can be attained by flow cytometry cell sorting, but alternative purification techniques were investigated as sorting by flow cytometry was time consuming and not easily accessible because there were no onsite facilities available. Magnetic bead sorting was therefore tested as a rapid alternative method that could be carried out under sterile conditions on site, therefore maximising the survival of sorted cells by minimising the delay between cells sorting and subsequent experiments.

Purification trials with direct one step labelling of tetramer-PE stained PBMCs with anti-PE beads resulted in poor recovery and purity of the selected cells. The reasons for that observation were likely to be two fold: firstly the columns used for bead purification were small making it less likely that all beads could be retained amongst a much greater majority of unstained cells; secondly, dead cells and monocytes were able to bind the tetramer reagent non-specifically and resulted in decreased purity of the selected fraction. Rather than opting for using two consecutive larger columns, which would have required a significant investment, a two step purification protocol was tested. Initially, $CD8^+$ T cells were purified from the total PBMCs by using a negative selection Kit (labelling and retaining all cells but $CD8^+$ cells), resulting in pure "bead free" $CD8^+$ cells being eluted from the column (Figure V.1, panels A and B). Then, the $CD8^+$ T cells were stained with CMV Tet-PE, and positively selected with anti-PE beads, and subsequently loaded onto a second small column.

Satisfying purity was obtained using this method: 84% of non gated total events acquired in the flow cytometry analysis of the purified population were Tet^+ in the case of a PBMC sample from Patient 1 (Figure V.1, panel C). This method was used to purify CMV specific T cells for Vβ repertoire analysis and comparing spectratyping analysis to a newly developed reference strand conformational analysis (RSCA, method allowing the hybridisation of the amplified Vβ DNA fragment to a control template and able to show a 1 base pair mismatch between the two) and showing the predominance

and profound expansion of a limited number of T cell clones (McGreavey *et al*, unpublished observations).

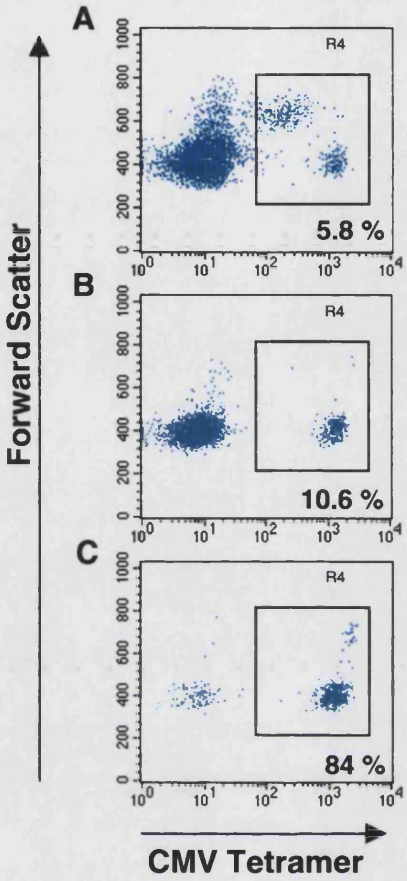


Figure V.1 Enrichment of CD8$^+$ Tet$^+$ T cells

Two step magnetic bead purification of antigen specific CD8$^+$ Tet$^+$ T cells. Stepwise enrichment of CD8$^+$ Tet$^+$ cells from a PBMC sample from Patient 1 shown with: Total PBMCs (panel A), CD8 negatively selected cells (panel B), and phycoerythrin (or Tet$^+$) positively selected CD8$^+$ cells.

The enrichment of high purity antigen specific T cells can be achieved by flow cytometry or magnetic bead sorting. However purified cells would still be bound to the tetramer reagent and to the antibody/bead complex in the case of magnetic sorting, even after the recommended washes. Although clinically approved bead selection is possible (i.e. CD34 selection), there is no reagent currently available that would allow Tet$^+$ positive cell selection. Furthermore, HLA/peptide tetramers are bacterial products, are not sterile unless filtered, and would need throughout testing before they can have a clinical use. A possible solution to this problem that could also prevent T cell activation would consist of removing the tetramer reagent from the cell surface after the sorting procedure.

2-iminobiotin HLA/peptide tetramers

2-iminobiotin was selected as an alternative to biotin in the preparation of HLA/peptide tetramers because of its low affinity for streptavidin at acidic pH compared to biotin. As the biotinylation reaction and the subsequent tetramerisation of the HLA/peptide monomers is performed at pH 8, the preparation of tetramer reagents with 2-iminobiotin was performed as described in Chapter II. Subsequent staining was identical to that performed with tetramer prepared with biotin (Figure V.2), except all steps were carried out at 4°C to avoid activation and internalisation of the complexes, and was visualised by flow cytometry. PBMCs stained with 2-iminobiotin CMV tetramer were then incubated for 10 min in PBS at pH 4.5 at 4°C with excess competing biotin to test if the tetramer binding could be disrupted at the surface of CMV specific T cells. Partial disruption of tetramer binding can be visualised with a lower Tet staining fluorescence intensity, indicating that fewer TCRs were bound per Tet^+ T cell (Figure V.2, panels B and D). This phenomenon was found to be reversible if the PBMCs were subsequently stained again with CMV Tet, confirming that the T cell surface remained intact after this acidic treatment (panel C). As described above, 2-iminobiotin tetramer staining and subsequent acid wash was not sufficient to remove some of these reagents completely from the cell surface. This clearly demonstrated that this approach to reversible HLA/peptide tetramer staining could give a satisfactory result, but that it needed to be improved to remove the tetramer reagents totally. In this system, the only parameters that could be modified to improve tetramer disruption would be to incubate the cells in a buffer at lower pH and/or for longer periods of time. However, due to lack of time at the end of this PhD thesis, these experiments could not be attempted. Moreover, although such drastic treatment would most likely remove more tetramer molecules from the cell surface, it might also be very toxic to the cells therefore reducing their viability and impair their possible use in subsequent adoptive transfer protocols.

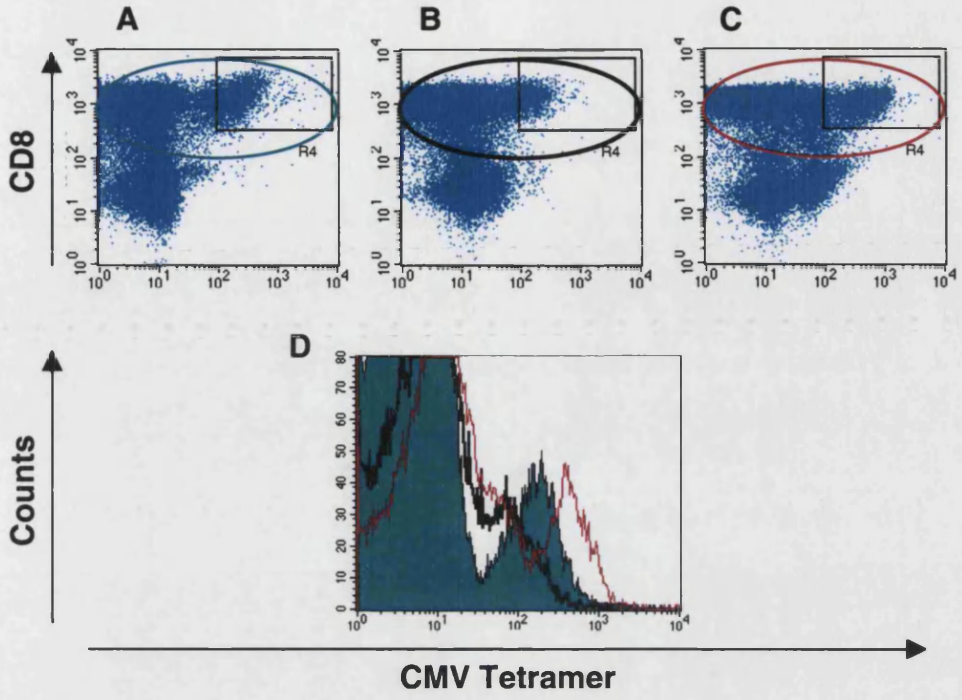


Figure V.2 2-iminobiotin HLA/peptide tetramer staining

Test stainings with 2-iminobiotin CMV tetramer. The CD8 versus Tet staining of the $CD3^+$ gated population is represented (panel A). Panel B represents the same PBMC sample labelled with 2-iminobiotin tetramer and 10 min washing in pH 4.5 buffer with competing biotin (competitive acid wash). The same sample was again the same sample after 2-iminobiotin tetramer staining, competitive acid wash and secondary staining with CMV Tet (panel C). Panel D represents the Tet staining histogram for each circled $CD8^+$ population (panels A, B and C).

A similar system was then described that allowed the removal of HLA/peptide tetramers after binding to antigen specific T cells by direct competition without requiring any modification of the pH conditions. This method required the encoding of a specific peptide residue, StreptagII, at the end of the HLA class I molecule (instead of the bsp2 tag as a target for subsequent biotinylation). The StreptagII peptide could bind streptavidin or StrepTactin (a mutated streptavidin molecule forming a polymer, Voss *et al*, 1997) with lower affinity than d-biotin. Therefore, incubating with a buffer containing free biotin could easily compete out StreptagII and cause the dissociation of the HLA/peptide polymer complex. The downside of this method was that both HLA class I and b2-microglobulin needed to be expressed with the StreptagII peptide and to be both bound to StrepTactin in order to obtain stable molecules (Knabel *et al*, 2002). This represented a better alternative to removing HLA/peptide tetramer reagents that

may be possible with 2-iminobiotin tetramers. Additionally, a new biotin analogue is now commercially available (DSB-X Biotin, Molecular Probes), can be used as an alternative to 2-iminobiotin and can be competed out by biotin without the requirement for an acidic pH. This would provide a reversible staining option that could be adapted to any established HLA/peptide tetramer synthesis protocol.

Although both these approaches have been designed to avoid TCR triggering, the specific ligand interaction of HLA/peptide tetramers with the TCR could be exploited to induce T cell activation and/or proliferation.

In vitro modulation of antigen specific T cell responses

HLA/peptide-antibody chimeramer specificity

The preparation and testing of HLA/peptide chimeramers was described in Chapter II. This demonstrated that these complexes were composed of successfully refolded HLA/peptide complexes and of mouse (or rat) antibodies. A series of HLA/peptide chimeramers were prepared in parallel to regular HLA/peptide tetramers diluted at equivalent volume to the chimeramers to take the added volume of the antibody into account, and were made simultaneously from the same batch of HLA/peptide monomers. This was done to ensure that tetramer and chimeramer reagents should represent the same number of HLA/peptide complexes for a given volume of reagent. The only difference between the chimeramer complexes therefore resides solely in the presence of the antibody.

The ligand specificity of these complexes was tested to verify that the high avidity to the TCR would be conserved without significant interference from the presence of the antibody molecule. CMV HLA/peptide tetramer, chimeramer and antibody stainings were performed on separate aliquots from the same PBMC sample. Representative stainings from patients 5 and 6 are shown on Figure V.3 (panel A) and were performed identically to regular stainings as described in Chapter II. The difference observed between tetramer and chimeramer stainings were found to be minimal, and control staining with the antibody alone demonstrated that the difference should have been very pronounced had the antibody interference been substantial. Triplicate stainings were carried out with tetramers and different chimeramers to further analyse this difference and determine if the antibody would interfere with the HLA/peptide-TCR specificity. A control chimeramer complex was prepared with an antibody to a mouse cell surface marker (anti-mouse CD8, gift from Dr. Zamoyska) which does not cross-react with the human molecule and therefore should not bind to human cells. It was added to the panel of chimeramers tested in this experiment. Parallel comparisons of sample stainings with high (Figure V.3, patient 6, blue) or average frequencies (HD1, red) of CMV Tet$^+$ T cells did not show significant differences. Additionally, PBMC samples from patient 6 were also stained simultaneously with both regular CMV Tet labelled with APC and with one of the following chimeramers labelled with PE: CMV Tet/28, CMV Tet/40L, CMV Tet/4-1BB, CMV Tet/95 and CMV Tet/3.

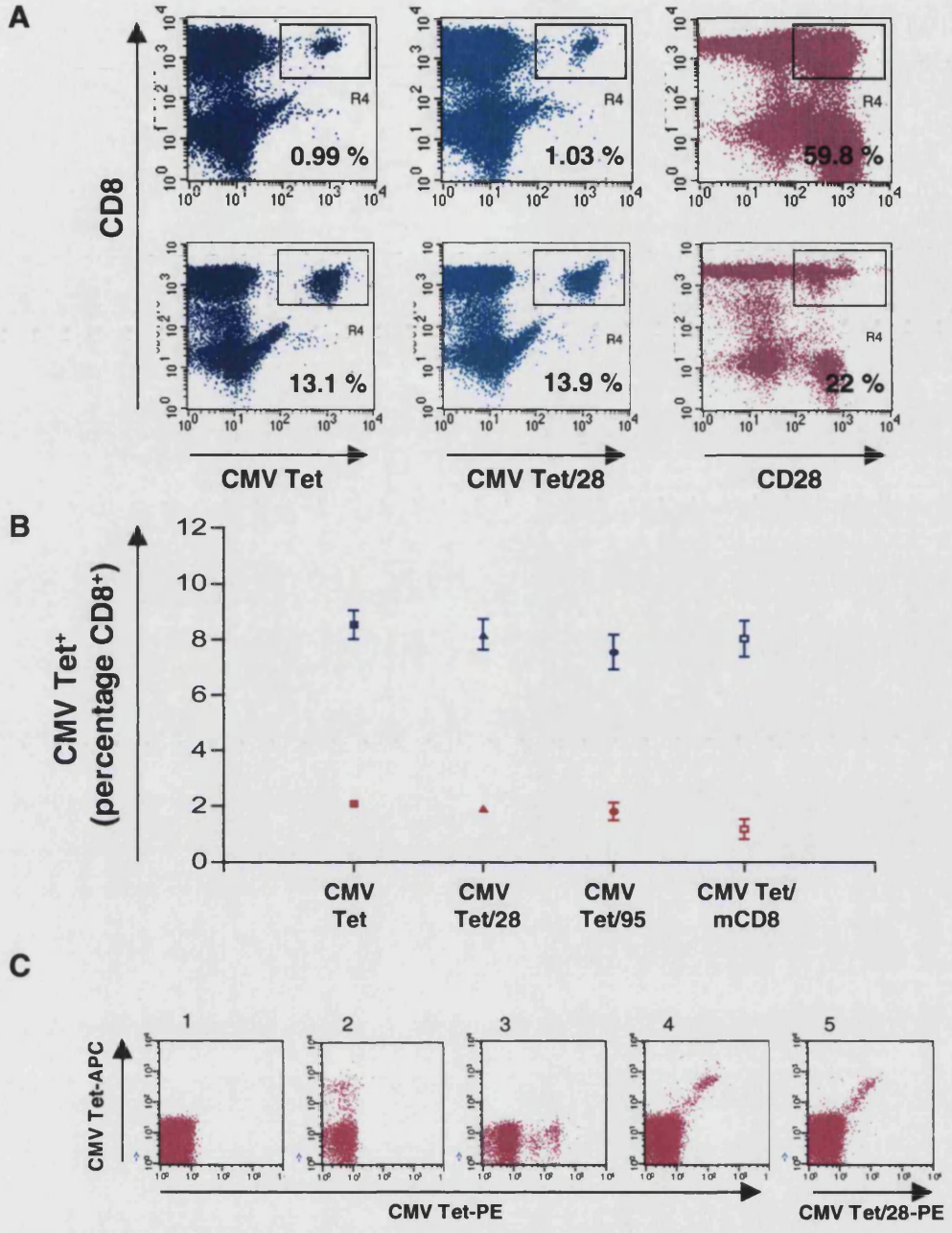


Figure V.3 HLA/peptide-antibody chimeramer staining

Panel A: Representative comparative stainings on two patients with HLA/peptide tetramer (●), HLA/peptide-anti-CD28 chimeramer (CMV Tet/28, ●) and anti-CD28 antibody alone (●). The percentage of gated $CD8^+$ cells is indicated at the bottom right of each dot-plot (patient 5,upper row; patient 6, bottom row).
Panel B: independent experiments comparing parallel triplicate stainings of PBMC samples from donors with high (patient 6, blue symbols) or average (healthy donor 1, HD1, red symbols) Tet^+ frequencies. Stainings were performed with CMV tetramer and the following CMV chimeramers complexes: CMV Tet/28, CMV Tet/95 with anti-CD95 antibody and CMV Tet/mCD8 with anti-mouse CD8 antibody; with mean Tet^+ frequencies and the standard deviation represented.
Panel C: representative stainings of PBMC samples from a single donor (patient 6) labelled with: 1, no label; 2, CMV Tet-APC; 3, CMV Tet-PE; 4,CMV Tet-APC and CMV Tet-PE; 5, CMV Tet-APC and CMV Tet/28-PE.

As seen on representative stainings on Figure V.3 (panel C), both reagents were able to bind to antigen specific T cells and no unspecific staining could be detected staining with the chimeramer alone (Figure V.3, panel 5). This result was consistent with all the chimeramer reagents tested, and separate comparative triplicate stainings with both CMV Tet-APC the different chimeramer reagents showed no difference in the frequency of antigen specific T cells that were stained by either reagent respectively. These results jointly indicated that the chimeramer reagents conserve their specificity for the TCR despite the presence of the antibody molecule. The capacity of theses complexes to activate antigen specific T cells was examined next to establish if they could be used for in vitro stimulation.

Activation of antigen specific T cells by HLA/peptide tetramer and chimeramer complexes

Upon triggering of the TCR, and during T cells activation, a signalling cascade is induced that results in the up-regulated expression of specific proteins. Some of these proteins, such as CD69 and CD25 are expressed at the cell surface and see their expression being up-regulated at relatively short time-points post activation. The up-regulation of the expression of both these activation markers was examined 24 hr after stimulation with tetramer or chimeramer reagents. Triplicate PBMC samples from a healthy CMV^+ individual were left unstimulated (no Tet control), stimulated with soluble CMV AE42 peptide, or stimulated with CMV tetramers or chimeramers. Chimeramer complexes were used individually (CMV Tet/28, CMV Tet/40L and CMV Tet/4-1BB) or in combinations consisting of equal parts with mixes of the following chimeramers: CMV Tet/28/40L, CMV Tet/28/4-1BB, CMV Tet 28/40L/4-1BB. The same volume of reagent was added for all stimulation experiments, which implied that the same level of TCR interaction with HLA/peptide molecules was applied, with different additional cross-linking of co-stimulatory molecules or combinations of molecules.

Tetramer and chimeramer reagents used for stimulation were composed of fluorescent-PE streptavidin. They were presumably internalised and degraded rapidly by T cell and/or PBMCs in culture as no residual fluorescence could be detected 24 hr after stimulation. Nevertheless, to discount any possible influence of remaining reagent in culture or of using tetramer complexes labelled with the same fluorescent molecule, subsequent stainings were performed with allophycocyanin labelled tetramer reagent (Tet-APC). Gated $CD8^+$ Tet^+ T cells were tested for their expression levels of CD69 and

CD25 respectively, and the proportion of Tet^+ T cells expressing high levels of these markers of activation was assessed (Figure V.4, panels A and C). Triplicate measurements for each stimulation condition were then plotted on bar graphs (Figure V.4, panels B and D) and indicated that the incubation of PBMCs with HLA/peptide tetramer or chimeramer reagents induced the up-regulation of both CD69 and CD25 expression at the surface of $CD8^+$ Tet^+ T cells. This confirmed that the stimulation of antigen specific T cells with HLA/peptide tetramer or chimeramer reagents resulted in the activation of the majority of these cells. For all stimulation conditions that were tested, the increase in expression of these activation markers was comparable. The presence of the antibody molecule on chimeramer complexes did not appear to impair their T cell activation properties.

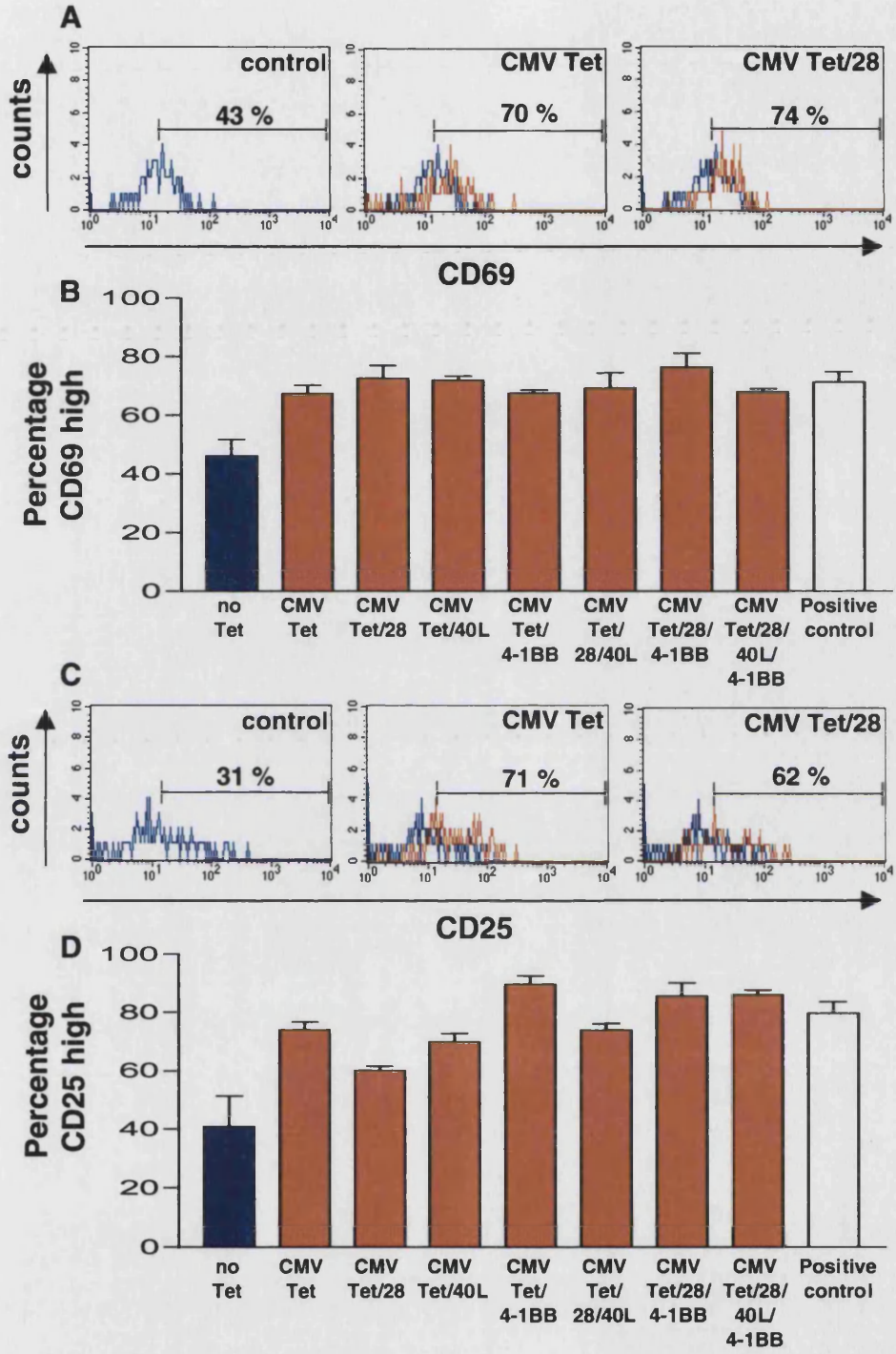


Figure V.4 *In vitro* stimulation with HLA/peptide tetramer and chimeramer complexes: expression of activation markers

Measurement of the up-regulation of CD69 and CD25 activation markers 24 hr after antigen specific stimulation. Panels A and C represent selected examples of measurements with no stimulation control (—), and the superimposed histograms for stimulated samples (—) graphs. Panels B and D represent the mean of triplicate tests (bars indicate the standard deviation) for control (no stimulation, ■), peptide pulsing (□), and each tetramer or chimeramer stimulation tested (■).

Slightly more pronounced differences were observed for CD25 expression than for CD69 expression, but this was difficult to interpret for several reasons: 1) in a repeat experiment, increases in CD25 expression were observed, yet the pattern of expression when comparing each stimulation condition could not be reproduced; 2) although the same number of PBMCs were plated in each well, this did not guarantee that the number of $CD8^+Tet^+$ T cells was the same between replicate wells. This might therefore result in a higher stimulus in wells with relatively less $CD8^+$ Tet^+ cells than in these with relatively more $CD8^+$ Tet^+ T cells per well; 3) the kinetics of CD69 and CD25 up-regulation upon T cell activation are different with the peak CD69 expression occurring earlier than the peak CD25 expression. As both CD69 and CD25 expression were measured at the same time-point post stimulation, the results presented here are indicative of up-regulated expression but do not necessarily represent a maximal response.

In order to compare the impact of the different stimuli on T cell activation more thoroughly, tetramer purified cells should be used to perform time-course measurements of activation markers. Another way of demonstrating the efficacy of cross-linking alternate receptors simultaneously to TCR triggering to specifically deplete Tet^+ cells by activating the programmed cell death pathway through the cross-linking of CD95. This would be in contrast to attempts at increasing and improving activation, but would provide evidence for antigen specific targeting together with inhibitory function.

Selective apoptosis of antigen specific T cells with anti-CD95 chimeramer reagent

Activated T cells express higher levels of the CD95 cell surface molecule than resting T cells. Subsequent interaction of CD95 ligand or anti-CD95 antibody with CD95 induces a cascade of signalling events that lead to programmed cell death or apoptosis.

As part of the assessment of the capacity of HLA/peptide chimeramer reagents to induce a targeted $CD8^+$ Tet^+ T cell co-signal, CMV Tet/95 chimeramers were prepared. An experimental protocol was established that allowed the measurement of the expression of the early enzyme markers of apoptosis: caspases (Apostat, as described in Chapter II). In an attempt to use a simplified experimental setting compared to using a PBMC sample from a healthy CMV^+ donor, a specific T cell line was obtained from colleagues in Leiden.

Two experiments were planned using these purified T cells: 1) the first experiment consisted of incubating the CMV specific T cells overnight alone, with CMV tetramer, with CMV Tet/95 chimeramer or with anti-CD95 antibody alone, then to assess the level of apoptosis after 24 hr in culture. This was to establish whether the CMV Tet/95 chimeramer complexes could be used to selectively deplete antigen specific T cell responses; 2) the second experiment involved using the same conditions as in the previous experiment, but adding the complexes to the CMV specific T cell line an hour prior to mixing them with fluorescently labelled and CMV peptide pulsed T2 targets, or adding them simultaneously by mixing with the target cells. The level of apoptosis would then have been measured in the CMV specific T cells as well as in the target population to establish if the use of CMV Tet/95 chimeramer complexes could specifically deplete CMV specific T cells and/or protect T2 cells from antigen specific T cell cytotoxicity.

However, only preparative experiments were completed (cell labelling and assessment of apoptosis) as the cell line that was obtained constitutively expressed high levels of CD95, high levels of caspases and showed poor viability in culture (~ 50%). These characteristics which made the experiments described above impossible, as the viability was not sufficient to carry out reliable tests. Despite the inability to demonstrate targeted apoptosis through binding of HLA/peptide molecules and cross-linking of CD95 molecules with HLA/peptide chimeramers in this particular experimental setting, the co-stimulatory effect induced together with the stimulation of antigen specific T cells with chimeramers could be assessed. This was achieved by testing the capacity of these molecules to induce specific proliferation of antigen specific T cells.

Proliferation of antigen specific T cells upon stimulation with HLA/peptide tetramer and chimeramer reagents

Upon TCR triggering and receipt of an appropriate agonist signal, a proportion of antigen specific T cells will undergo cell division. A similar experiment to that described previously with the activation of antigen specific T cells, stimulating the same number of PBMCs from a healthy CMV^+ individual in triplicate per stimulation condition, and assessing the proliferation of $CD8^+$ Tet^+ T cells in relation to $CD8^+$ T cells. To ensure that the proliferation observed in these assays was truly antigen specific and not somewhat influenced by any residual reagent present in culture, PBMCs were

stimulated with CMV tetramer and chimeramer reagents composed of streptavidin-PE, whereas stainings were performed with CMV tetramers prepared with streptavidin-APC. However as mentioned previously, no residual PE fluorescence could be observed in the PBMC samples 24 hr after stimulation.

The relative frequency of $CD8^+$ Tet^+ T cells was measured on day 0 (stimulation day, this acted as the baseline control) and after 7 days in culture following stimulation (Figure V.5, panels A and B). The results obtained after all stimulation settings were plotted and compared to a control where PBMCs only received identical amounts of IL7 and IL15 cytokines at the same time-points as stimulated samples (Figure V.5, panel C). After 7 days in culture, the relative frequency of $CD8^+$ Tet^+ T cells decreased in the control sample, whereas a vigorous increase could be observed in all stimulated samples. This data is representative of two independent experiments carried out with PBMCs isolated from the same CMV^+ healthy donor. The highest increase was observed by stimulating PBMCs with CMV Tet/28 with a mean difference of 5.5% between the frequencies measured on day 0 and 7 days after stimulation. Interestingly, all samples stimulated with HLA/peptide chimeramer complexes showed more pronounced increases than the samples stimulated with HLA/peptide tetramer complexes. In this experimental setting, it was not possible to strictly compare each different stimulation condition as the initial number of $CD8^+$ Tet^+ T cells may not have been identical in each individual well tested, as suggested by the high standard deviation values. However, the mean frequency increase was found to be consistently higher in all samples stimulated with HLA/peptide chimeramer reagents compared to these stimulated with HLA/peptide tetramer reagents, which supports the idea that cross-linking of cell surface co-stimulatory molecules had occurred and had promoted antigen specific $CD8^+$ Tet^+ T cell proliferation.

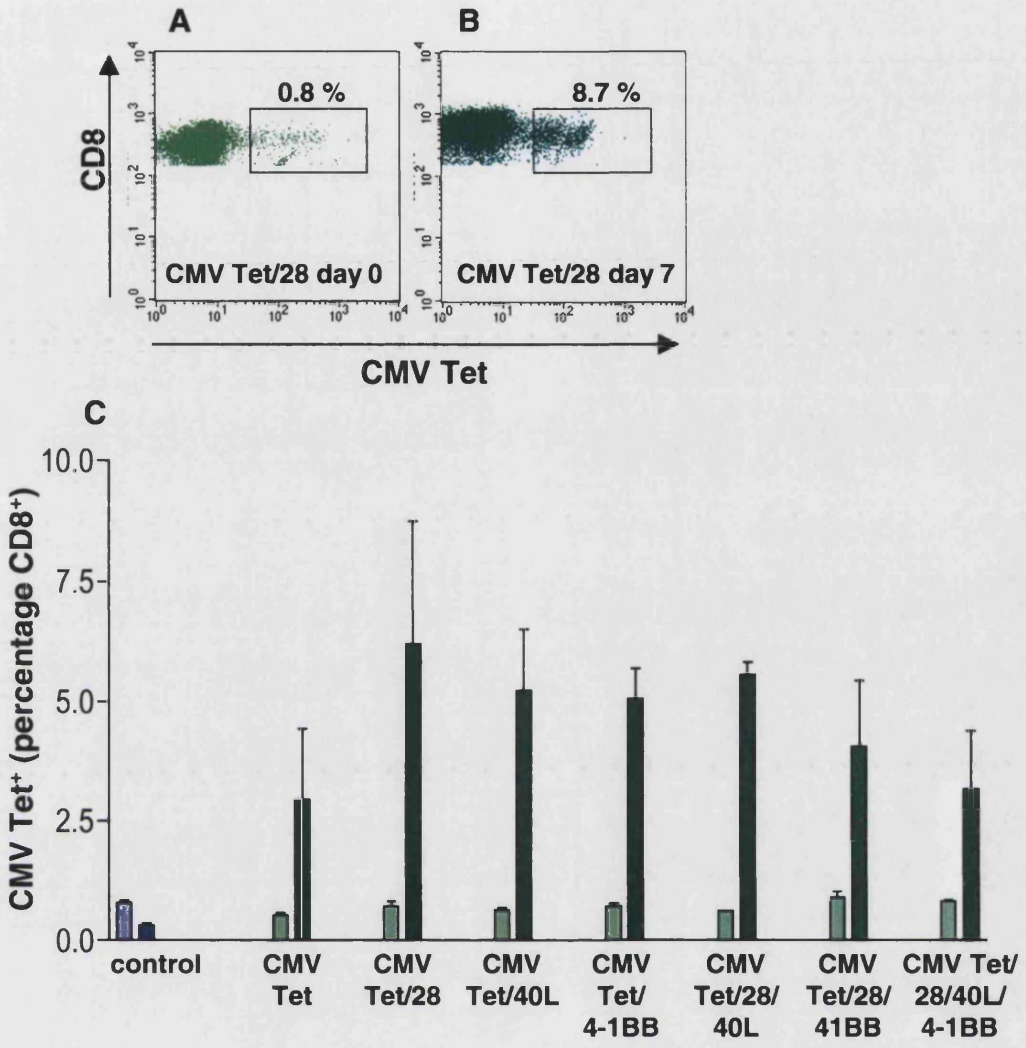


Figure V.5 *In vitro* stimulation with HLA/peptide tetramer and chimeramer reagents: proliferation of antigen specific CD8⁺ T cells

PBMCs from a healthy CMV⁺ healthy donor (HD1) were plated in triplicate and samples were stimulated with (■) CMV tetramer or chimeramer reagents or not stimulated (■). Assessment of the relative proliferation was assessed after 7 days in culture. The light bars represent day 0 and the dark bars represent day 7 after stimulation; error bars represent the standard deviation between triplicate samples.

The samples were further stimulated *in vitro* to establish whether they could be further expanded, but after 3 rounds of stimulation under the same conditions as described previously, all cells from the samples stimulated with chimeramer reagents prepared with the anti-CD28 antibody had died (CMV Tet/28, CMV Tet/28/40L, CMV Tet28/4-1BB and CMV Tet/28/40L/4-1BB) while all other samples had mean frequencies very similar to the original control sample (Figure V.5, panel C, light blue bar). The

frequencies measured in the samples that retained viability were: no Tet, 0.6%; CMV Tet, 0.8%; CMV Tet/40L, 1.19%; CMV Tet/4-1BB, 0.68%. This observation was somewhat anecdotal, but supports the concept that these complexes induce different levels and/or types of stimulation, and perhaps that CD28 co-stimulation/activation might promote activation induced cell death.

Since the ability to effect TCR triggering together with co-stimulation has only been examined with respect to the activation and the proliferation of antigen specific $CD8^+$ T cells following tetramer or chimeramer stimulation, it was considered that further phenotypic markers need to be studied, in order to establish the properties of these reagents *in vitro* and at later stages *in vivo* and their impact on T cell activation, proliferation and differentiation.

Phenotypic characteristics of tetramer and chimeramer stimulated antigen specific T cells

The successful stimulation and proliferation of antigen specific T cells with HLA/peptide tetramer and chimeramer complexes were further examined in conjunction with the phenotypic markers as described in Chapter III. PBMCs were isolated from a healthy CMV^+ donor (blood sample, HD1) and either left unstimulated, stimulated with CMV tetramer, with CMV chimeramer (Tet/28, Tet/40L, Tet/4-1BB, Tet/28/40L, Tet/28/4-1BB or Tet/28/40L/4-1BB) or with CMV peptide. For each stimulation condition tested, CMV Tet^+ events were gated (panel A, Figure V.5). The same marker combinations as these mentioned in Chapter III were used with CD45RO, CD27 and CCR7 stained in parallel to CMV tetramer staining. The effect of the *in vitro* stimulation on the expression of these markers was tested 7 days after stimulation and cell culture.

The first point that was evident from this data was the presence of Tet^+ cells expressing high or low levels of TCR molecules at their cell surface, as visualised by high or low tetramer fluorescence staining intensity on panel A. If compared to the control cells without any antigenic stimulation, tetramer stimulated and particularly peptide stimulated PBMCs comprise a majority of low TCR CMV specific T cells. This observation may be indicative of a higher stimulation threshold for these cells if they were to face a subsequent antigenic challenge as there would be less density of cell surface ligand available for the binding of HLA/peptide molecules. Remarkably, PBMCs stimulated with CMV chimeramer reagents showed a different profile of TCR expression levels with the presence of both high and low TCR expressing cells, detected

in higher numbers than in the control sample. All CMV chimeramer complexes tested but Tet/28/40L/4-1BB produced a similar phenotypic profile as seen for Tet/28 on Figure V.6. This indicated that antigen specific T cells stimulation with chimeramer reagents induced the proliferation of both high TCR and low TCR cells, which could be a significant advantage in the case of a secondary challenge. Additionally, the combination chimeramer Tet/28/40L/4-1BB induced less intense proliferation, as seen on Figure V.5 and Figure V.6, but the majority of these cells after 7 days in culture expressed high levels of TCR molecules at their surface, which would make them sensitive to a secondary antigenic challenge.

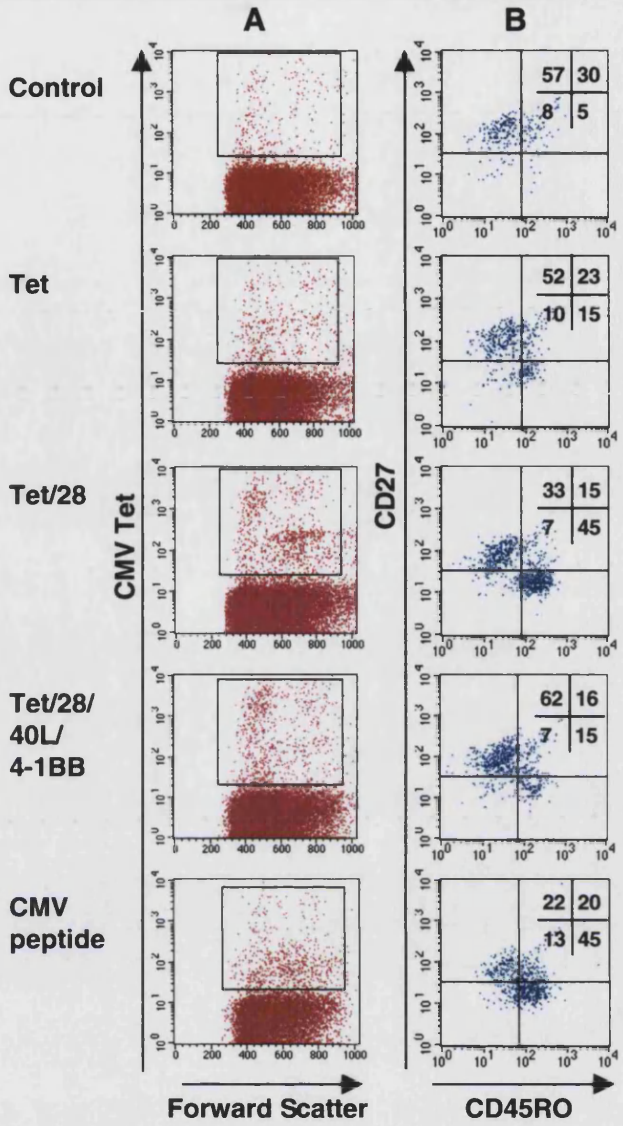


Figure V.6 CMV tetramer and chimeramer stimulated antigen specific T cells: CD27 and CD45RO expression

Panels show CMV Tet stainings of PBMCs from a healthy CMV$^+$ donor (HD1) unstimulated, after stimulation with CMV Tet, CMV Tet/28, CMV Tet/28/40L/4-1BB, or CMV AE42 peptide and cultured for 7 days. Tet$^+$ T cells were gated (panel A,●) and the cells were sub-categorised as function of their CD27 and CD45RO expression (panel B,●). The relative percentages of events in each sub-category are reported in the top corner of each panel.

When the phenotypic markers of these Tet gated cell populations were examined, no changes in CCR7 expression could be detected compared to those previously seen in Chapter III with the majority of Tet^+ cells and $CD8^+$ cells being $CCR7^-$. However, differences in CD45RO and CD27 expression, were seen depending on the stimulation condition examined (panel B, Figure V.6). Whilst the majority of the cells in the control sample were either of resting/quiescent phenotype ($CD45RO^-$ $CD27^+$) or of memory/activated phenotype ($CD45RO^+$ $CD27^+$), stimulated cells had a larger proportion of cells with a more differentiated phenotype and a higher proportions of effector memory type cells ($CD45RO^+$ $CD27^-$). The most differentiated phenotypes for CMV specific T cells were observed in the case of peptide pulsed PBMCs with the least resting/quiescent cells remaining compared with other stimulation conditions, and the most effector memory/activated and terminal effector ($CD45RO^-$ $CD27^-$) cells detected after 7 days in culture. This may reflect the fact that peptide pulsing only triggers the proliferation and differentiation of memory/activated and effector type cells, but that resting/quiescent cells might be merely maintained or kept cycling and might not be stimulated to proliferate and/or differentiate under these conditions. Stimulation with CMV specific tetramer and chimeramer reagents maintained and/or seemed to induce a significant proportion of resting/quiescent T cells or antigen inexperienced cells, which would be an important component in the response to a further CMV specific stimulus. It was clear from the results that certain co-stimulatory chimeramers appears to favour the expansion of resting/quiescent type $CD8^+$ CMV Tet^+ T cells whilst conserving their phenotype and inducing little effector/memory differentiation or proliferation. By contrast, the highest proportion of effector memory cells is seen when PBMCs are stimulated with Tet/28 or with peptide. In the case of stimulation with Tet/28 chimeramer, we can observe after 7 days in culture the expansion of both resting/quiescent type cells as well as memory/activated/effector type cells. Although it is likely that the resting/quiescent phenotype cells have proliferated compared to the control sample, it is also possible that these cells have survived better in culture with a higher cell death rate in the control. The stimulation with Tet/28 chimeramer was comparable to all other chimeramer stimulation conditions tested with the exception of Tet/28/40L/4-1BB (Figure V.6 and data not shown).

Consequently, further study of combined antigen specific stimulation and co-stimulation using HLA/peptide chimeramer reagents might be useful to determine to which degree each co-stimulatory molecule contributes to survival, to proliferation, to the generation of differentiated memory and effector cells. However the results

discussed above only represent a single experiment and would therefore need to be repeated to confirm the phenotype results obtained.

The finding that antigen specific T cells, in particular naïve T cells could be stimulated to differentiate and/or proliferate were studied further in the context of primary CMV responses to test if tetramer and/or chimeramer complexes could be used to trigger antigen specific immunity.

Induction of CMV specific primary immune responses

Three healthy CMV^- donors (HD3, HD4 and HD5) as well as three CMV^- recipients of SCT with CMV^- donors were selected. In all PBMC samples used, CMV Tet^+ T cells were found to be below the detection limit of the assay. PBMCs were plated in triplicate wells and either left unstimulated, stimulated with CMV tetramer or were stimulated with CMV Tet/28 chimeramer. The frequency of Tet^+ T cells present in the samples was assessed after two or three rounds of stimulation and cell culture in the presence of IL7 and IL15. The Tet/28 chimeramer reagent was chosen on the basis that it might provide the two signals thought to be necessary for the induction of a T cell response 1) signalling through the TCR with the HLA/peptide molecule; 2) the CD28 co-stimulatory signal. It was not possible to test the other chimeramer reagents combinations due to limiting cell numbers. A PBMC sample from a CMV^+ SCT patient with a CMV^+ donor (Patient 4) was also stimulated and represented a positive control. In this particular case (Figure V.7, panel B), CMV specific Tet^+ T cells increased after 2 rounds of stimulation but went back to control levels after 3 rounds of stimulation. This demonstrated that Tet^+ cells could be stimulated and proliferate *in vitro* following a protocol identical to that was used previously to stimulate Tet^+ cells from a CMV^+ healthy donor (Figure V.5).

In the case of the stimulation of PBMCs from CMV^- healthy individuals, no increase in the frequency of CMV specific T cells was detectable in tetramer or chimeramer stimulated wells compared to control wells (Figure V.7, panel A) following 2 rounds of stimulation. However, after 3 rounds of stimulation, a significant increase in the frequency of CMV specific Tet^+ CTLs could be detected when compared to the controls. In Addition, this increase was more pronounced in wells stimulated with CMV Tet/28 chimeramer reagent. As these results were consistent in all 3 CMV^- healthy individuals tested, they further supported the concept of an enhanced stimulating capacity of Tet/28 chimeramer complexes compared to standard tetramer complexes

bearing the same HLA/peptide combination. It can be noted that a significant proportion of the increase in the HD5 samples occurred with the sole addition of IL7 and IL15 cytokines without antigenic challenge as can be seen with a high frequency of $CD8^+$ CMV Tet^+ T cells in the control. This is likely to represent the proliferation of T cells other than CMV specific that may be activated at the time of culture set-up and express high levels of CD28 on their cell surface, therefore would be more sensitive to cross-linking of CD28 and to cytokine exposure. This unexpected high increase in the case of HD5 may be explained by a high level of susceptibility to allergens in this individual, and raises questions about the suitability of stimulation in individuals with inflammatory reactions with the need for further testing and/or purification of the wanted T cell population after stimulation in similar cases.

However the general consistency of these stimulation results in all 3 CMV^- healthy individuals was very encouraging, and constituted an alternative method to raising antigen specific T cell responses without recourse to infectious material. Therefore the PBMCs that were left over for each stimulation condition were cryopreserved and stored so that cytotoxicity and phenotyping assays could be performed later. Unfortunately due to lack of time at the end of this PhD project, these tests could not be performed.

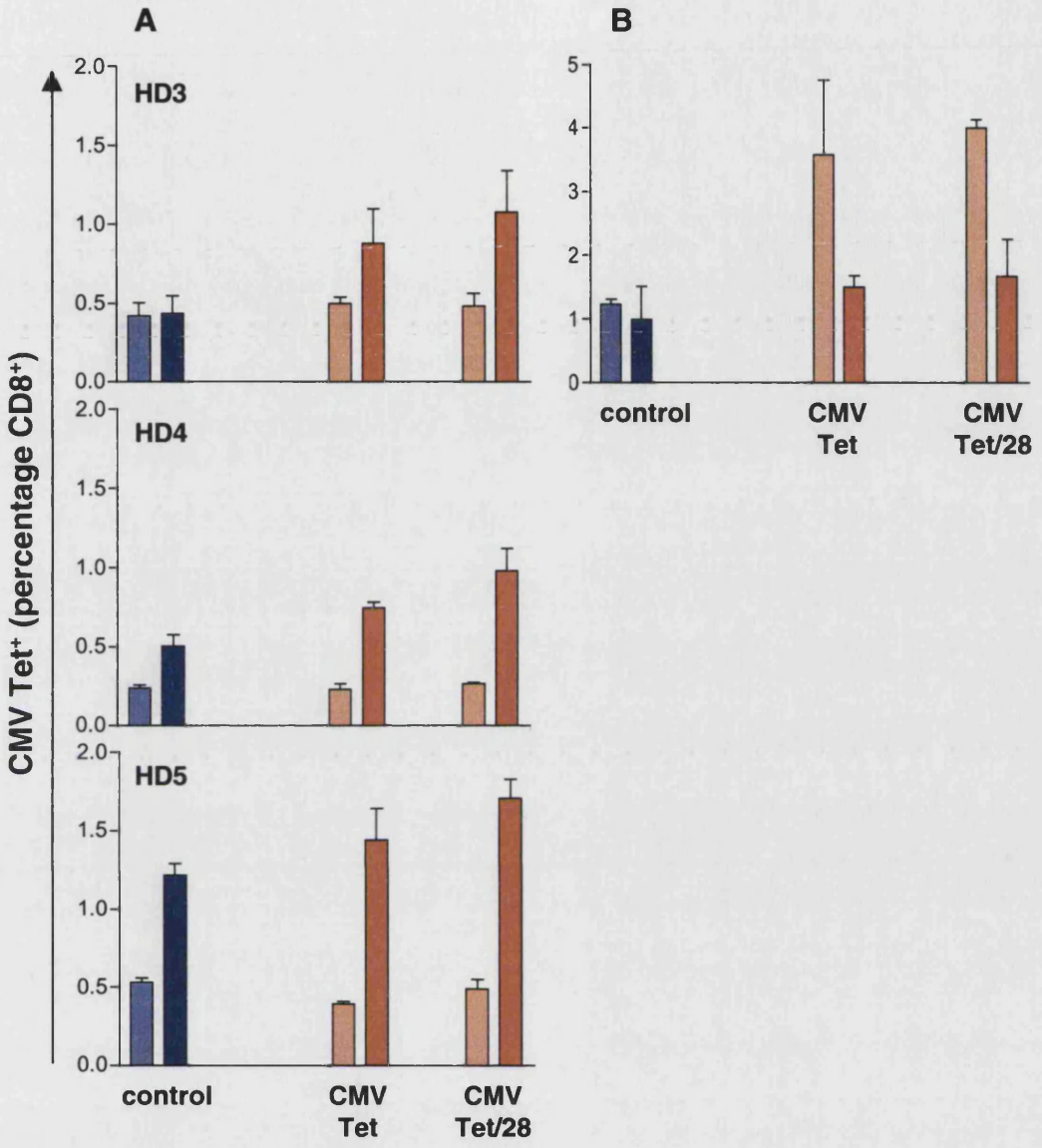


Figure V.7 *In vitro* stimulation with HLA/peptide chimeramers: primary specific immune responses

3 CMV^- healthy individuals were selected and PBMC isolated and three aliquots prepared. These correspond to: culture alone (control, ■ and ■) or stimulated with HLA/peptide CMV specific tetramer (CMV Tet) or chimeramer (CMV Tet/28) reagents (■ and ■, panel A). The frequency of $CD8^+$ Tet^+ T cells was measured after 2 rounds (light colour) or 3 rounds (dark colour) of stimulation and culture. Error bars indicate the standard deviation. The positive control sample comprised PBMCs from a CMV^+ SCT patient 4.

In the case of CMV^- SCT recipients with CMV^- donors, no differences were seen between control and stimulated PBMCs after 2 or 3 rounds of stimulation (data not shown). This may reflect the fact that immune reconstitution is slow after SCT and also

that few naïve T cells, which would be needed to provide a primary response, are transferred in the transplant procedure. Furthermore, few naïve T cells are effectively generated especially in adult patients because of decreasing thymic activity with age and/or toxicity of the transplant procedure as well as immunosuppression post transplant in some cases. Nevertheless, it was demonstrated in Chapter IV that CMV^+ recipients with CMV^- donors are able to raise CMV T cell responses *in vivo*. In one particular case (patient 1), it was possible to test the origin of these CMV specific Tet^+ CTLs by specific Y chromosome fluorescence *in situ* hybridisation of $CD8^+$ Tet^+ sorted cells (Figure V.1), as this patient had a female donor. The cells were confirmed to be of donor origin with this technique. This confirmed that it was clearly possible to generate primary type CMV specific responses in vivo after SCT with a CMV^- graft. Therefore the stimulation protocol described above would need to be improved and further tested to determine if an anti-CMV CTL immune response could be induced in vitro in samples from CMV^- donors and might be used for transfer to CMV^+ recipients.

As CMV specific T cells could be stimulated following specific TCR triggering with CMV tetramer or CMV Tet/28 chimeramer reagent, this raised the possibility that they could also be used to boost specific T cell responses. This was tested in the context of tumour CML specific T cell responses.

Stimulation of CML specific T cell responses

As CML specific T cells could be detected in a proportion of CML patients, the possibility of boosting this response by increasing their numbers using HLA/peptide-antibody chimeramers was examined. Cryopreserved PBMC samples from 8 CML patients were selected in order to stimulate CML specific T cell responses as these patients were HLA-$A*0301^+$ $b3a2^+$. As was shown in Chapter III, CMV specific T cells can be detected in some patients. Therefore this raises the possibility that a memory type immune response might be stimulated in this cohort of patients. A sample from a CMV^+ SCT recipient who was not treated for CML but was HLA-$A*0301^+$ (patient 6) was also tested in this experiment. This would act as a control sample, and also would determine the possibility of triggering CML specific responses in the context of a CML naïve subject (this has been shown to be possible in healthy donors, see Bocchia *et al*, 1996; Norbury *et al*, 2000). As cell numbers were limiting, it was not possible to test a large number of conditions for stimulation, or to test multiple wells per setting. Predominantly, it was only possible to set up a control and 1 stimulation variable using

the CML Tet/28/40L/4-1BB reagent. This reagent combination was chosen as it might provide the most diverse stimulus, as well as possibly inducing the proliferation of less differentiated antigen specific T cells that consequently may be better suited for a repetitive or durable antigenic stimulus, as would be the case for an established tumour.

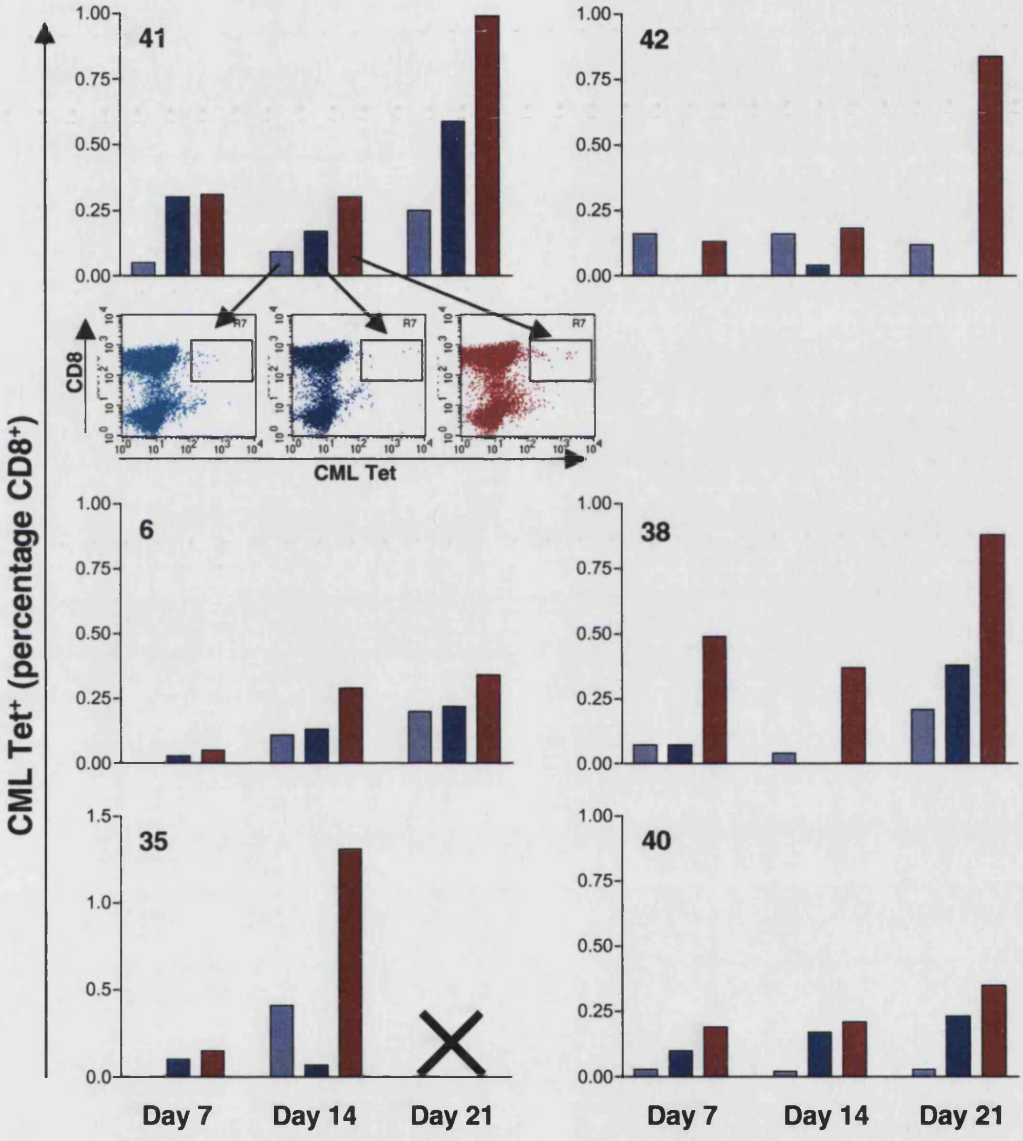


Figure V.8 *In vitro* stimulation of tumour specific T cell responses with HLA/peptide chimeramer reagents

5/8 HLA-A*0301$^+$ b3a2$^+$ CML patients and 1 HLA-A*0301$^+$ b3a2$^-$ CML$^-$ patient (patient 6) are shown. PBMC samples were stimulated with CML Tet/28/40L/4-1BB. The relative proliferation of CD8$^+$ Tet$^+$ T cells was assessed by CML tetramer staining 7, 14 and 21 days after stimulation and culture (■). The lack of data due to cell death is indicated by a black cross (patient 35). The following controls are represented: no chimeramer stimulation followed by CML tetramer staining (■), chimeramer stimulation followed by no tetramer staining (■).

For 5/8 CML patients, an increase in the proportion of CML Tet^+ T cells was observed after 2 or 3 rounds of stimulation (Figure V.8). In all of the cases where an increase could be detected, these were somewhat discrete but specific and represented a relatively small number of antigen specific T cells compared to the negative control stainings, as can be seen on the example dot plots, accompanying the graph for patient 41. It should be noted that not all patients were receiving the same treatment: patients 38, 41 and 42 were treated with the tyrosine kinase inhibitor Imatinib Mesylate, whereas patients 35 and 40 were treated by SCT. This might suggest that CML specific T cell responses may be boosted in a proportion of patients, which could then promote a tumour specific immune response, independently of the treatment received. For 3/8 CML patients, no $CD8^+$ CML Tet^+ T cell could be detected above the levels of the controls used (data not shown). This might indicate that in these patients, no KQSSKALQR peptide specific T cells were present or that they could not be stimulated by the experimental conditions used in this assay. However, the absence of $CD8^+$ CML Tet^+ T cells or their presence in low numbers in the 5 samples where amplification could be detected is not altogether surprising as immune responses to established tumours are notoriously difficult to generate and/or stimulate. Additionally, CML specific Tet^+ T cells appeared to increase also in the PBMC sample from patient 6, who was transplanted to treat a haematological malignancy other than CML, which may be indicative of a primary response, and/or of the proliferation of naïve T cells.

Although these results are preliminary because only a single sample could be tested per condition per patient and the chimeramer reagent used and/or the culture conditions may not be optimal, they nevertheless represent a promising novel method to stimulate tumour specific T cells *in vitro*. Although these assays are preliminary, they warrant repetition, along with further characterisation and testing of the CML specific T cells to establish their specific cytotoxicity. This strategy deserves to be investigated further as it may provide new immunotherapeutic opportunities.

Discussion

As was shown in the previous chapters, the technical advance brought about by the use of HLA peptide tetramer complexes has been very significant for the study of antigen specific T cell responses. Understanding the kinetics of the CTL immune response to a specific pathogen has been especially useful in the context of SCT, and to tumour antigens in oncology, with in some cases the absence or apparent deficiency of responses. These have allowed for the development and impact assessment of the treatments used in patients and in studying the contribution of the immune system.

However applications for HLA/peptide tetramer complexes can be taken further and be used as a specific tools for the purification of antigen specific T cell populations for further characterisation (for example Vβ repertoire analysis) and/or adoptive transfer. This provides a direct way of modulating antigen specific responses *in vivo* by the direct addition of antigen specific T cells by infusion in peripheral blood or at the specific site where the response is needed. Despite the fact that these areas have been developed relatively recently, a number of in vivo applications have been reported to date, for example for the prevention of CMV infection in individuals "at risk" after SCT (Einsele *et al*, 2002) or to target tumour to which antigen specific responses have been described such as melanoma (Dreno *et al*, 2002; Dudley *et al*, 2002; Meidenbauer *et al*, 2003). These adoptive transfer studies are still limited in numbers, therefore the full potential and impact of such therapies has yet to be formally established for each clinical setting where they are applied on a sole basis or in combination with conventional therapy. In the clinical applications cited above, the HLA/peptide tetramer complexes used were synthesised and used with regular streptavidin and biotin molecules as described in Chapter II. No significant adverse effect have been reported after transfer of tetramer purified antigen specific T cells. In most cases, transferred T cells are shown to proliferate after transfer and to be effective towards their specific HLA/peptide restricted target. The majority of these transfer protocols involve a period of *in vitro* culture prior to transfer, but the impact of tetramer purification with possible TCR down-regulation after the purified cells get activated through the purification process, as well as the rate of possible activation induced cell death is not known. Although, the benefits of reversible tetramer staining in the context of adoptive transfer of antigen specific T cell concerning the parameters described above have not been formally studied, they may be of significant impact for the study of TCR and/or co-stimulation cross-linking and their effect on resting T cells together with the signalling

pathways involved. Therefore the use of 2-iminobiotin tetramers or analogue molecules that allow reversible staining without TCR triggering may prove to be an advantage in some experimental settings.

Yet the very fact that HLA/peptide tetramer complexes are able to specifically trigger T cell activation can also be exploited to modulate antigen specific T cell responses. This was attempted in vitro in this chapter with the use of HLA/peptide tetramer or HLA/peptide-antibody chimeramer reagents. Specific binding of the tetramer and chimeramer reagents to a specific antigen restricted T cell population was demonstrated after staining PBMCs. This point was very important in the case of the chimeramer reagents as it meant that both TCR activation and co-stimulation could both be delivered specifically to the antigen specific T cell population of interest. Although this was shown in the context of a well established antiviral response, this may be crucial to the generation or boosting of responses in more difficult cases such as anti-tumour responses. As the combination of endogenous MHC and co-stimulation were shown to contribute to T cell recognition (Wülfing *et al*, 2002), this may prove to be an advantage that chimeramer complexes have over regular tetramer complexes for the modulation of an antigen specific immune response.

Clearly, both HLA/peptide tetramer and chimeramer complexes were capable of triggering T cell activation and proliferation, with better results obtained with chimeramer complexes. This is in agreement with the concept cited previously, and also with the fact that if given a strong signal, no co-stimulation is required for the activation and proliferation of $CD8^+$ T cells (Wang *et al*, 2000), but that co-stimulation may augment these. The finding that there was no statistical difference between the activation or proliferation of antigen specific T cells induced with the different chimeramer complexes was unexpected, as one might consider that the contribution of each co-stimulatory molecule tested in TCR signalling and T cell activation would differ. However in the CMV model that was studied in this chapter, there may be several limiting factors in the detection of these possible differences. Firstly the use of a relatively high dose of tetramer or chimeramer reagent might be limiting, therefore masking the contribution of co-stimulation with a very potent TCR signal; secondly, the fact that the AE42 peptide is a potent agonist might render co-stimulation dispensable to generating a response. It may therefore be possible to better assess these differences in the CMV model by studying a T cell line or clone and testing dose dependent responses to tetramer and chimeramer stimulation. Furthermore, different models using a weaker

agonist response or a lower avidity complex might be better suited to highlighting these differences. Consequently modulation of antigen specific responses using HLA/peptide tetramer or chimeramer complexes offer new avenues for stimulation protocols. They also may constitute new tools for assessing what the requirements for generating antigen specific T cell responses are, as well as the respective effect of different co-stimulatory stimuli applied together with TCR triggering.

As opposed to specific stimulation of antigen specific T cells, HLA/peptide complexes have been shown to inhibit certain responses (that may be of interest for example in the case of allogeneic responses) if used in a low avidity complex form such as a dimer. This was shown in a transgenic mouse model (O'Herrin *et al* (2001). This further makes the point that the requirements for activation by positive modulation and tolerisation by weak interaction or targeted specific depletion need to be better established to induce the desired effect in an *in vitro* or *in vivo* protocol.

The important differences that could be established between peptide, tetramer and chimeramer stimulation were that the level of TCR down-regulation and the level of differentiation of the $CD8^+$ CMV Tet^+ T cells may be affected by the type of stimulus that was chosen. The fact that TCR down-regulation varies between experimental settings may have a significant impact on the subsequent susceptibility of these cells to further antigen specific stimuli. This is due to the fact that TCR becomes down-regulated after T cell activation (Cai *et al*, 1997) and remains marked for internalisation after dissociation from MHC (Coombs *et al*, 2002), this therefore might result in durable down-regulation of the number of TCR molecules present at the cell surface. As fewer TCR would be effectively available for binding at the cell surface, a much stronger stimulus would be needed to achieve activation of the targeted T cell. Additionally, the fluorescence intensity of tetramer binding has been shown to correlate with T cell avidity and anti-tumour activity (Yee *et al*, 1999), therefore *in vitro* stimulation protocols may benefit from the selection of a stimulus that while inducing proliferation might also maintain or re-induce high levels of T cell expression at the T cell surface. *In vitro* stimulation and proliferation of antigen specific T cells needs to be assessed to determine what the differentiation levels of the generated cells is prior to use in *in vitro* experiments or adoptive transfers, in order to maximise their efficacy and life-span. This may contribute to the improvement of adoptive transfer strategies where although the transferred cells were shown to specifically kill their targets *in vitro*, this might not be representative of their *in vivo* activity (Monsurro *et al*, 2002). Despite the

fact that some differentiated memory cells might revert to less differentiated phenotypes while maintaining their functions (Van Leeuwen *et al*, 2002), there is also a possibility that these cells might reach high frequencies *in vitro* but reach replicative senescence (Oxenius *et al*, 2001). Yet they may remain in peripheral blood for long periods of time by default of apoptosis (Spaulding *et al*, 1999), which might after transfer constitute an over-representation and or compete out other responses. This could therefore be detrimental to a treated patient in the longer term, especially if large numbers of antigen specific T cells were transferred. This phenomenon can be seen in elderly individuals with large numbers of antigen specific T cells specific for latent viruses such as CMV or EBV. A good proportion of these cells are not functional, and their domination of the T cell pool is thought to be associated with a higher incidence of infections by other agents and earlier mortality in CMV and/or EBV seropositive individuals with large responses compared to seronegative aged matched controls (Ouyang *et al*, 2003). However this last point may not be of immediate concern for SCT recipient "at risk" of CMV infection or indeed for cancer patients.

The new possibilities of inducing primary and/or boosting specific T cell responses using artificial antigen presenting cells or complexes such as the chimeramer reagents described in this chapter (or other recently described non-cellular artificial antigen presenting cells consisting of latex beads coated with HLA/peptide tetramer complexes, CD80 and CD54 molecules (Oosten *et al*, 2002)) offer attractive improvements for the advance and the wider application of T cell immunotherapy.

Chapter VI

General Discussion and Conclusion

In the present PhD thesis, successful detection, monitoring and modulation of antigen specific immune responses was demonstrated. These parameters have proven to be of interest in the context of haematological malignancies, where their characterisation might benefit patients by improving previously described antigen specific T cell responses.

The first strong argument in favour of using such a system as HLA/peptide multimer complexes is their intrinsic quality of receptor specificity. This guarantees that the T cells which are targeted are truly specific to the HLA/peptide combination chosen for detection or modulation, therefore avoiding binding to other irrelevant T cells bearing a receptor of different specificity. This is especially important in the SCT setting as it is crucial to determine that the antigen specific immune reconstitution that is occurring is acting against tumour or virus restricted targets whilst avoiding one of the major post SCT complications, GvHD (caused by the presence of host reactive donor T cells) that would be exacerbated by a non-specific stimulus. The results presented in Chapter III showed the successful detection of an immunodominant anti-CMV CTL response after testing several possible candidate HLA/peptide combinations. With a different approach, CML specific HLA/peptide complexes were detected at the surface of CML b3a2$^+$ cells, and HLA/peptide multimers then allowed the detection of T cells restricted to that particular HLA/peptide combination in peripheral blood from CML patients. As illustrated in these two separate applications, HLA/peptide multimers can be used to test for the presence of and then to characterise antigen specific CD8$^+$ T cell immune responses for new HLA/peptide combinations. Other applications of HLA/peptide tetramer or multimer reagents include the possible detection of antigen specific helper T cells (CD4$^+$ HLA class II/peptide specific complexes), or of antigen specific T cell in tissues rather than in peripheral blood for example. These applications as well as other currently well defined procedures using HLA/peptide and MHC/peptide complexes were the subject of a detailed review issue of the Journal of Immunological Methods (2002; issue 268,1). Applications reviewed in this journal also include the

possibility of discovering previously unknown ligands for less common HLA molecules such as non-classical HLA molecules (for example HLA-E). Although the specificity of HLA/peptide multimers for their relevant T cell receptor constitutes great advantages to their use, it can also constitute a limitation to their application in the detection, monitoring and modulation of antigen specific responses for several reasons. Firstly, it requires a knowledge of the target antigenic peptide. Therefore, the use of HLA/peptide multimer complexes would be restricted to individuals possessing the HLA molecule used in the HLA/peptide multimer, which would restrict the number of individuals that may be targeted by such studies or therapies. Furthermore the HLA frequency distribution of rare alleles and the high degree of HLA polymorphism would both contribute to these limitations. However, not all HLA polymorphisms affect the peptide binding capabilities to some extent within a subgroup of HLA alleles, so that the limitations presented above might be overcome by selecting the predominant HLA alleles in the majority of individuals from the ethnic population studied. Another important aspect to keep in mind is that even in the case of a well described immunodominant response (i.e. the HLA-A*0201/AE42 CMV specific CTL response), the detection of antigen specific immune responses with HLA/peptide multimers represents a fraction of the total immune response. This may be especially relevant in the case of an anti-tumour response where several tumour-associated and/or minor histocompatibility antigen specific responses are believed to contribute to the anti-tumour immune response. Further, the diversity of the CTL immune response restricted to a particular pathogen or tumour is also likely to be diversified to avoid or counter immune escape mechanisms. Additionally, the diversity of the response would also be influenced by the different combinations of HLA molecules present at the cell surface, as well as by the viral strain or translocation type of the specific viral or tumour antigen targeted. Consequently, it is likely that a combination of HLA/peptide complexes composed of common HLA molecules in a selected patient population together with several important antigenic peptide targets might provide optimal detection, monitoring and modulation. For example, comparable CTL responses restricted to either the pp65 or the IE1 CMV protein but not both could be detected in distinct HLA-A*0201 individuals with different other HLA alleles combinations, providing some evidence that certain major HLA molecule combinations might favour the processing and/or presentation of an immunogenic protein over another (Khan *et al*, 2002; Paston *et al*, personal communication). Such HLA/peptide multimer "cocktails" containing several HLA/peptide complexes might be developed for routine clinical use in the detection

and/or purification (if produced in detachable form with 2-iminobiotin or analogue) of antigen specific T cell pools that would include a range of known common HLA molecules with their restricted major antigenic peptides within a defined ethnic population. However, the need for multiple antigenic peptides binding to a single selected HLA allele might not be necessary in the case of a well-defined immunodominant CTL response.

In an immunodominant T cell response, such as the HLA-A*0201 restricted response to CMV pp65, it is possible to monitor antigen specific T cell responses, as was demonstrated in Chapter IV of this thesis. This strategy was used successfully in the clinic to assess the progress of SCT patients and their antigen specific immune reconstitution, together with the detection and quantification of CMV infection. This study is the first to formally establish a positive correlation between high levels of CMV specific cytotoxic T cells and protection from CMV infection. This was particularly useful to establish the response of patients to various treatments as well as the relative influence of these treatments on the CMV specific immune response. The present study, as well as other studies of larger patients groups allowing multivariate analyses, that are perhaps more adequate in the context of SCT, for the identification of factors influencing CMV infection. One of these factors included the occurrence of CMV specific T cell immunodeficiency and lymphopenia induced by steroid treatment and could be used as predictors of CMV infection, recurrent and/or late CMV infection, which are all linked to higher mortality in these patients (Aubert *et al*, 2001; Nichols *et al*, 2001; Boeckh *et al*, 2003). These findings would support the hypothesis that immunosuppression induced to prevent or treat GvHD might be in most cases where late CMV infection is observed, immunosuppression is the cause of CMV specific T cell immunodeficiency resulting in CMV infection. By contrast, one might argue that in the case of early CMV infection likely due to a yet insufficiently reconstituted immune system, the pro-inflammatory properties of CMV might contribute to the occurrence of GvHD. Furthermore, evidence from a mouse SCT model (Cooke *et al*, 1998) indicates that the transplant conditioning and procedure themselves can cause significant local trauma in the lung resulting in inflammation. This may be linked to alloreactive T cells to find sanctuary in this organ and not others, where cells are known to be susceptible to CMV infection and/or reactivation. Thus the transplant procedure itself might be at the origin of either or both GvHD and CMV infection. Consequently, the relative possible causative effects of CMV infection, GvHD and lung pathology on each other might remain unclear in the context of SCT as these appear to be connected. This specific

question apart, the study presented in this thesis clearly demonstrated the value of monitoring CMV specific immune reconstitution in parallel to CMV viral load determination. As the study develops to include a larger number of patients with the description of new HLA/peptide combinations, this strategy will enable better patient adapted treatments with regards to CMV infection and perhaps also to GvHD treatment. Similar strategies with CML might identify factors or treatments that enhance the anti-tumour immune response in the case of CML relapse for example.

The use of HLA/peptide multimers to monitor antigen specific immune reconstitution after SCT also identified settings such as heavily T cell depleted transplants as is the case for unrelated and/or HLA mismatched transplant, where immune recovery is slow and inadequate and represents a risk for CMV infection. In these cases, patients might benefit from the adoptive transfer of CMV specific T cells. As shown in Chapter V, it is now possible to select purified populations of antigen specific T cells using HLA/peptide multimer reagents. As mentioned previously, it is important to consider that most of these applications are somewhat limited to a minority of patients because of the restriction caused by HLA/peptide specificity. However, a number of in vivo trials along these lines have already occurred. Although there has not yet been any evidence of adverse effects from the use of HLA/peptide multimer reagents for the purification of antigen specific T cells followed by adoptive transfer, it may be desirable (if only to avoid TCR triggering, or sensitisation to the complexes when repeat transfer might be needed) to synthesise complexes that can be separated from the T cell they were bound to prior to transfer or use. This has become possible with the use of lower affinity biotin analogues in the synthesis of HLA/peptide multimers that can be easily competed out by conventional biotin, therefore offering a more versatile system.

Furthermore, HLA/peptide multimers, by their very composition, can trigger T cell activation in an antigen and HLA restricted manner. In Chapter V, the option of including a biotinylated antibody to a T cell co-stimulatory molecule was tested as a new way of enhancing T cell activation and proliferation *in vitro*. These modified HLA/peptide-antibody chimera multimer complexes or chimeramers were shown to have maintained their antigen specificity. The influence of stimulation, induced by different chimeramer complexes, was tested by assessing the activation and proliferation of antigen specific T cells. This was achieved successfully, and although all chimeramers tested could induce higher levels of both specific T cell activation and proliferation than tetramers, it was not possible to establish consistent differences

between the former complexes. This was likely due to the fact that PBMCs were used as targets for stimulation rather than an antigen specific T cell line or clone. The complexes used in this study were composed of biotinylated antibodies to co-stimulatory molecules, but it would also be possible to use a biotinylated soluble form of the co-stimulatory ligand itself. However, results from these experiments are valuable as they also demonstrate the specificity of these complexes and their restricted action on antigen specific T cells in a complex cellular mixture that may be closer to a physiological setting than experiments conducted with T cell lines or clones might be. Some of the most significant applications for the use of these complexes *in vitro* in the context of SCT would be in the first instance to use chimeramers as "minimalist" antigen presenting molecules for the induction of a primary immune response. Unfortunately, there was not enough time at the end of this thesis to confirm the cytotoxic properties of T cells obtained with this method, but these experiments are currently under way. This may become an extremely valuable technique for the development of adoptive T cell transfer from non-immune donors, such as for CMV^+ SCT patients with CMV^- donors for whom adoptive transfer is not currently a possible treatment option. These complexes may also find applications in enhancing the potential of an anti-tumour immune response in vitro that after adoptive transfer or perhaps direct infusion might contribute to breaking the tolerance to the tumour and participating as well as enhancing the anti-tumour immune response. Although a lot more work needs to be completed before such therapeutic strategies can be applied to *in vivo* settings, HLA/peptide-antibody chimeramer complexes also offer exciting new ways of examining the joint effects of T cell receptor triggering and of specific co-stimulation. A very wide range of studies could be envisaged using these complexes, for example: determining antigen dependent activation threshold requirements for primary or memory T cell responses; testing the relative importance of co-stimulation in the case of low affinity peptide or low affinity HLA/peptide-TCR interaction; or testing their effect on activation induced cell death or T cell proliferation and survival are amongst the many possibilities.

Appendix

Study proposals:

CMV study consultant Information sheet

GP/(Consultant) INFORMATION SHEET

Title of Project

Monitoring of CMV antigen specific T cells in Bone marrow transplant patients before and after transplant.

We at the Anthony Nolan Research Institute and Haematology Dept of the RFH are currently embarking on a project to investigate the reconstitution of the immune system after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections but of specific importance to transplant patients is CMV. The precise details of how fast these cells regenerate and how effective they are in fighting CMV virus in Bone marrow transplant patients still remains unclear. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood These samples would be taken at the same time as the regular checks occuring at the hospital and will not require additional venepuncture.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and then subsequently at the same times when blood is being taken for routine monitoring of CMV status, this is usually twice weekly during the early phase post transplant. Monitoring will continue at outpatient appointments for up to 9 months following transplant.

DONORS- blood is required once at the pre-donation health check.

The cells extracted from these samples will only be used for the research project that the patient has agreed to take part in.

The proposed study will use class I soluble HLA molecules in the form of tetramers. These will be used to specifically detect the presence of circulating antigen specific T cells in the peripheral blood of patient who reactivate CMV following BMT.
CMV viraemia is a significant problem in immunosuppressed patients following BMT, and before their new haemopoetic system has been able to fully develop to provide the appropriate level of immune response to counteract the occurrence of CMV infection de novo or through the reactivation of pre exisiting CMV. Monitoring the immune potential of a patient to mount a specific immune response to a virus such as CMV, will allow us to obtain a better understanding of what is happening to the CMV specific T cells in patients who reactivate CMV post BMT or who develop de-novo CMV viraemia following BMT. The acquisition of such data will allow us to correlate the ability of patients to resolve CMV with the concurrent expansion or development of CMV specific CD8 T cells in their peripheral circulation. The analysis of such information has implications for improved therapy or clinical intervention at specific time points resulting in improved treatment of patients developing CMV disease post BMT.
The role of CMV specific CD8+ T cells in the resolution of CMV viraemia is well documented. However specific details about the dynamics of expanded CMV +ve populations the relative relevance of CD4+ T cells to the action of CD8 antigen specific cells and whether CD8 population expansions are productive or unable to act specifically to remove CMV positive cells in BMT patients are all questions which need answering. In addition the contribution of chimerism to the cellular dynamics of antigen specific T cells in BMT patients is also an important question that needs to be investigated.

CMV study patient Information sheet

PARTICIPANT INFORMATION SHEET

Title of Project

Monitoring of CMV antigen specific T cells in Bone marrow transplant patients before and after transplant.

We at the Anthony Nolan Research Institute are currently embarking on a project to investigate the reconstitution of the immune system after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections but of specific importance to transplant patients is CMV. The precise details of how fast these cells regenerate and how effective they are in fighting CMV virus in Bone marrow transplant patients still remains unclear. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood (which is approximately an egg cup full). These samples would be taken at the same time as the regular checks you will have at the hospital and will not require you to have any extra venepunctures.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and at the same times when blood is being taken for routine monitoring of CMV status, this is usually twice weekly during the early phase post transplant, you will then be monitored at your outpatient appointments for up to 1 year following transplant.

DONORS- blood is required once at your pre-donation health check.

The cells extracted from these samples will only be used for the research project you have agreed to take part in.

If you are willing to take part in this study and agree to provide the samples requested, your medical records will also be examined by researchers from The Anthony Nolan Bone Marrow Trust's Research Institute, who are outside the employment of the NHS. All information will remain confidential.

CML study consultant Information sheet

GP/(Consultant) INFORMATION SHEET

Title of Project

Monitoring of BCR-ABL antigen specific T cells in Bone marrow transplant patients before and after transplant.

We at the Anthony Nolan Research Institute and Haematology Dept of the RFH are currently embarking on a project to investigate the reconstitution of the immune system after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections but also possibly in combatting emergent leukaemia cells. These cells are of specific importance to transplant patients post grafting . The precise details of how fast these cells regenerate and how effective they are in fighting residual leukaemic cells in Bone marrow transplant patients still remains unclear. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients before and after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood These samples would be taken at the same time as the regular checks occurring at the hospital and will not require additional vene-puncture.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and then subsequently at the same times when blood is being taken for routine monitoring of CMV status, this is usually once weekly during the early phase post transplant. Monitoring will continue at outpatient appointments for up to 9 months following transplant.

DONORS- blood is required once at the pre-donation health check.

The cells extracted from these samples will only be used for the research project that the patient has agreed to take part in.

The proposed study will use class I soluble HLA molecules in the form of tetramers. These will be used to specifically detect the presence of circulating BCR-ABL antigen specific T cells in the peripheral blood of patient before and after BMT and during episodes of GVH.
Relapse can be a significant problem in immunosuppressed patients following BMT, and before their new haemopoetic system has been able to fully develop to provide the appropriate level of immune response to counteract the occurrence of residual leukaemic cells through the regrowth of pre exisitingleukaemic progenitors that have escaped conditioning. Monitoring the immune potential of a patient to mount a specific immune response to residual leukaemic cells and during relapse, will allow us to obtain a better understanding of what is happening to the levels of anti leukaemia specific T cells in patients who relapse, during DLI, and during episodes of GVHD post BMT. This will be compared with levels of these cells in patients pre transplant. The acquisition of such data will allow us to correlate the ability of patients to resolve relapse as a result of DLI with the concurrent expansion or development of leukaemia specific CD8+ T cells in their peripheral circulation. The analysis of such information has implications for improved therapy or clinical intervention at specific time points resulting in improved treatment of patients relapsing post BMT.
The role of anti leukaemia specific T cells in the resolution of relapse following DLI is well documented. However specific details about the dynamics of expanded leukaemia specific populations the relative relevance of CD4+ T cells to the action of CD8+ antigen specific cells and whether CD8 population expansions are productive or unable to act specifically to remove leukaemia positive cells in BMT patients are all questions which need answering. In addition the contribution of chimerism to the cellular dynamics of antigen specific T cells in BMT patients is also an important question that needs to be investigated.

CML study patient Information sheet

PARTICIPANT INFORMATION SHEET

Title of Project

Monitoring of BCR-ABL antigen specific T cells in CML patients before and after Bone marrow transplant.

We at the Anthony Nolan Research Institute are currently embarking on a project to investigate the presence of naturally occurring anti leukaemic white cells present in the immune system before and after bone marrow transplantation. As you will be aware, the immune system is severely depleted after pre-transplant conditioning. Research has shown that specific cells in the normal immune system are responsible for combating many virus infections and also possibly malignant cells such as leukaemic cells. These cells are of specific importance to transplant patients with leukaemia. The precise details of how fast these cells regenerate and how effective they are in fighting residual leukaemic cells in Bone marrow transplant patients still remains unclear. Also unclear is why these cells fail to function properly before transplant thus allowing leukaemia to develop. This project uses newly developed techniques which will provide valuable information about the levels and activity of these cells in patients before and after bone-marrow transplantation.

To help us carry out this work we require 20mls of blood (which is approximately an egg cup full). These samples would be taken at the same time as the regular checks you will have at the hospital and will not require you to have any extra venepunctures.

TRANSPLANT RECIPIENTS- blood is required prior to transplant and at the same times when blood is being taken for routine monitoring of CMV status, this is usually once weekly during the early phase post transplant, you will then be monitored at your outpatient appointments for up to 1 year following transplant.

DONORS- blood is required once at your pre-donation health check.

The cells extracted from these samples will only be used for the research project you have agreed to take part in.

If you are willing to take part in this study and agree to provide the samples requested, your medical records will also be examined by researchers from The Anthony Nolan Bone Marrow Trust's Research Institute, who are outside the employment of the NHS. All information will remain confidential.

Consent form

Consent forms for both studies were based on the same model:

PARTICIPANT CONSENT FORM

TITLE OF PROJECT

Monitoring of BCR-ABL antigen specific T cells in CML patients pre and post Bone marrow transplant and during DLI therapy

The patient should complete the whole of this sheet themselves.

Have you read the Patient Information Sheets? **YES / NO**

Have you had an opportunity to ask questions and discuss this study? **YES / NO**

Have you received satisfactory answers to all your questions? **YES / NO**

Have you received enough information about the study? **YES / NO**

Who have you spoken to? Dr / Mr / Mrs_____

Do you understand that you are free to withdraw from the study at any time, without having to give a reason for withdrawing and without affecting your future medical care? **YES / NO**

Do you agree to enter your this study and in doing so give permission for the researchers to have access to your medical notes? **YES / NO**

Do you give permission for us to notify your GP that you have entered this study? **YES / NO**

Do you agree to enter in this study? **YES / NO**

Patient s name _____
(in block letters)

Signature _____ Date |__|__|__|

Dr s signature _____ Date |__|__|__|

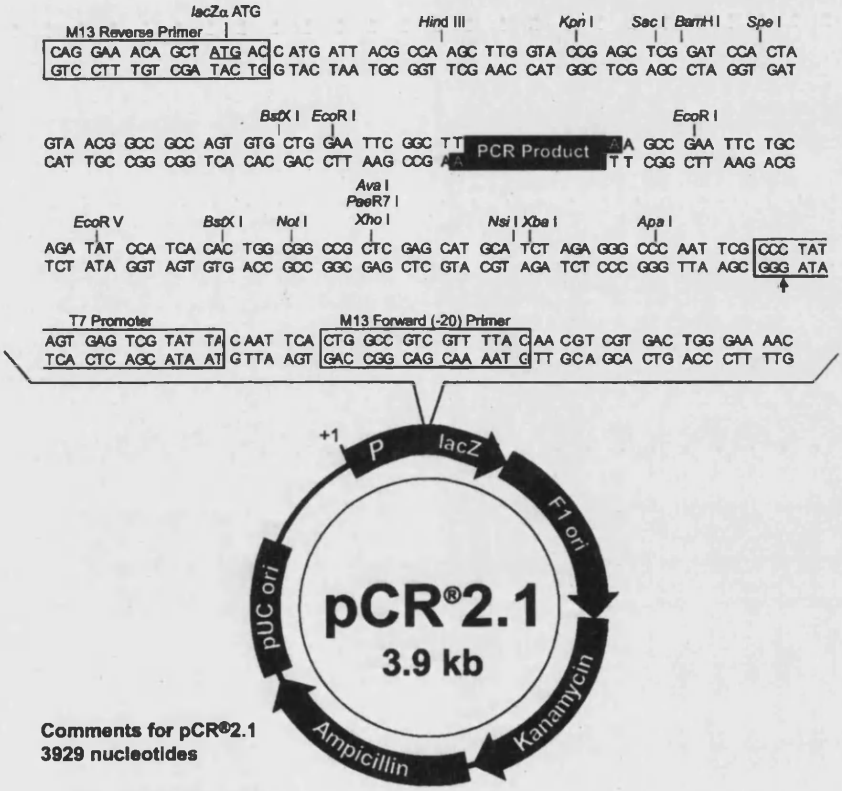
Dr s name _____
(in block letters)

File one copy in patients notes, one copy in CRF and one copy to patient.

Vector maps

PCR 2.1 vector, Invitrogen

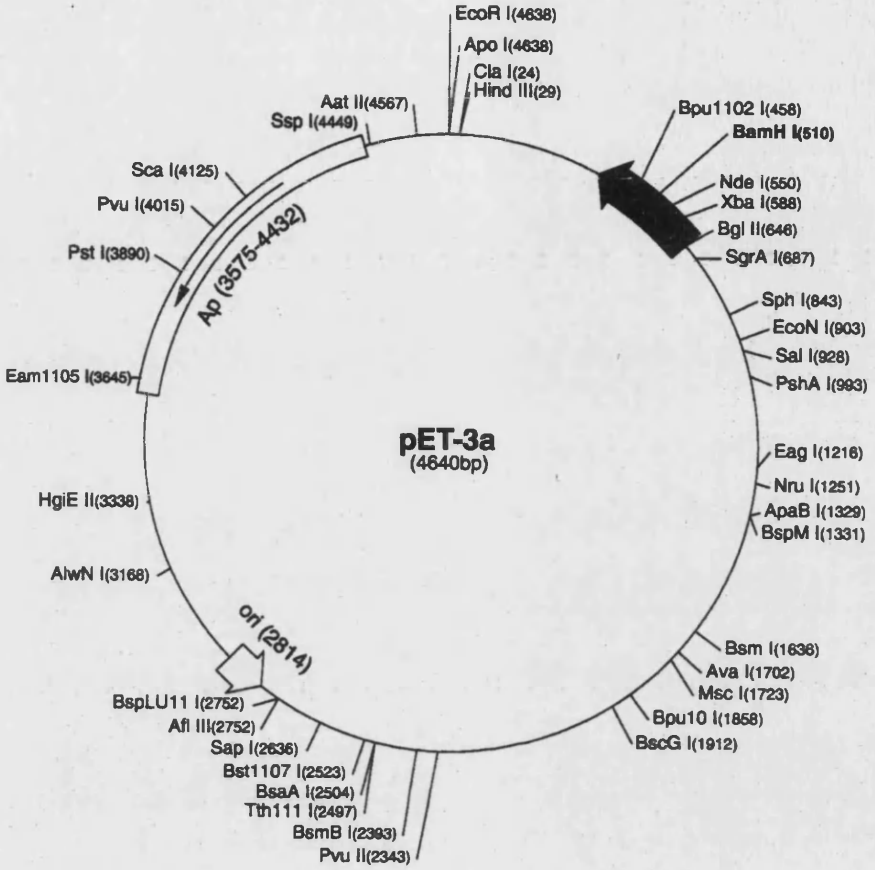
Map of pCR®2.1 The map of the linearized vector, pCR®2.1, is shown below. The sequence of the multiple cloning site is shown with a PCR product inserted by TA Cloning®. *Eco*R I sites flank the inserted PCR product on each side. The arrow indicates the start of transcription for the T7 RNA polymerase. **The complete sequence of pCR®2.1 is available from our World Wide Web site (www.invitrogen.com) or by contacting Technical Service (page 20).**

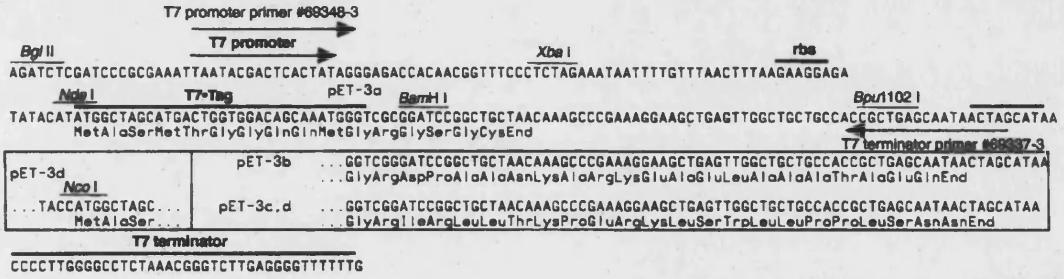


Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
Multiple Cloning Site: bases 234-355
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

pET 8 vector, commercially available pET 3d map, Novagen:





pET-3a-d cloning/expression region

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